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Address of the President Sir William Bragg, O.M., at the Anniversary Meeting, 30 November 1939

It is my first duty to refer to Fellows who have recently passed away.

GEORGE ALBERT BOULENGER was a student of the University of Brussels, his native city. He began at a very early age the study of reptiles, batrachians and fishes in the Natural History Museum at Brussels, and in 1880 became assistant naturalist there. In 1882, he was appointed a first class assistant in the Department of Zoology in the British Museum; he held this position until he retired in 1920.

He has been described as a man of tremendous energy, an extremely methodical mind, and an amazing memory. These qualities were the foundation of the vast amount of work which he accomplished. He was one of the most distinguished of the descriptive biologists who have brought fame to the British Museum during the past hundred years. His most important contributions to science were the series of elaborate and detailed monographs, mostly published by the Museum. These still remain the basis of modern systematic work. They dealt with the groups of life which had interested him from the beginning, the batrachians, fishes, lizards, snakes and so forth. Thanks to his energy as a collector and as a stimulator of collectors, the Museum is rich in its possessions of these types; for example, it possesses an unparalleled collection of fresh water fishes.

Boulenger was elected a Fellow in 1894 and served on the Council from 1903 to 1905. He was for some years a Vice-president of the Zoological Society and received many honours at home and abroad. He was naturalized in this country.

Towards the end of his long life he forsook his reptiles and for twenty years devoted himself to the study of roses.

WILLIAM McDOUGALL (1871–1938) was educated at a private school until he was 14, when he went for a year to a Real-Gymnasium at Weimar. The next four years were spent in the University of Manchester where he graduated with first class honours, making geology his special subject in his last year. He went to Cambridge in 1892, and obtained a 1st Class in both parts of the Natural Sciences Tripos, taking Physiology and Human Anatomy in Part II. He obtained the medical degree in 1897.

In 1897, while holding a house appointment at St Thomas's Hospital, he joined the Cambridge Anthropological Expedition in the Torres Straits. After a while he left the expedition to work with Dr Charles Hose on a study of the head-hunting tribes.

On his return to England his researches reverted to the physiological. He did much work on colour vision and took a great interest in the current controversies between the theories of Helmholtz and Hering. His investigations were made in the border country between sense physiology and the psychology of special senses; he insisted on the necessary correlation of the two subjects. Gradually he found most to do on the psychological side; his election to the Society in 1912 may be said to mark in time when he was rather on this side than the other. He held a post in Sully's department at University College (London) until 1904 when he was appointed to the Wilde Readership in Mental Philosophy at Oxford. McDougall drove a French ambulance in the earlier years of the war. Subsequently he was attached to the R.A.M.C. and put in charge of nervous patients: a duty for which he was especially fitted. At the same time it provided much material for his own line of work.

In 1920 he accepted an invitation to Harvard University; in 1927 he joined the staff of Duke University, North Carolina. He made a great name for himself in America. When I attended recent celebrations at Duke University on behalf of our Society I was much impressed by what was said to me of the esteem in which he was held and the value placed upon his work.

He himself considered *Body and Mind*, published in 1911, as his best written work: and its title may be taken to indicate the field of science in which he was eminent.

By the sudden death of GEORGE BARGER, at the early age of 60 years, science has lost a chemist of great distinction, whose personal and scientific influence spread far beyond this country. After a distinguished University course at Cambridge, Barger began his work in research while teaching Botany for a year in the University of Brussels. Then came a period as Chemist to the Wellcome Physiological Laboratories, where research on the active substances in ergot, from which came so much of unexpected biological interest, helped to give a Biochemical turn to Barger's activities. In later years he wrote a brilliant and scholarly monograph on ergot, dealing with all aspects of knowledge concerning that curious drug. After holding several academic appointments for shorter terms, he became in 1919 the first Professor of Chemistry in relation to Medicine in the University of Edinburgh, and held the Chair till 1938, when he became Regius

Professor of Chemistry in the University of Glasgow. Though his range was a wide one, he always regarded himself as essentially an organic chemist, and the researches of his own choice were on the stable end products of vital activity, rather than on the chemical changes involved in life itself. He received the Davy Medal of the Society a month before his death. Bilingual from his earliest years though British by birth, schooled in the major European tongues in Holland, Barger retained throughout his life a zest for learning new languages, and used them to make scientific and personal friendships in many countries. To an unusual degree he became an international figure in science, striving with honest enthusiasm to promote understanding and friendship among men of science throughout the world. He was elected a Fellow in 1919 and served on the Council from 1930 to 1932.

ALFRED WILLIAM PORTER, born in 1863, was educated at the University Colleges of Liverpool and London. He was a Fellow of University College London and Assistant Professor of Physics for many years, until in 1923 he was appointed to a University Chair. He retired in 1928.

Porter was essentially a man of learning: he was gifted also with the power of clear statement. His wide knowledge and his generous readiness to place it at the service of others made him the subject of frequent appeals for assistance. He had a penetrating and critical mind, and was particularly at home in the discussion of the fundamentals of physics and chemistry. He was especially interested in the border subjects between these two sciences, and his Presidency for a few years of the Faraday Society was well suited to his knowledge and abilities. His translation (with Carey Foster) of the Joubert treatise on Electricity and Magnetism and his new edition of Preston's *Theory of Light* were considerable developments of the originals, and being so gave him good opportunity for the use of his powers of construction and exposition. He gave a remarkably interesting address on "The Volta effect and kindred subjects" as President of Section A of the British Association, when it met at Glasgow in 1928.

He was the highly respected teacher of large numbers of University College students. He also did great service to physics in various administrative capacities: he was secretary to the University of London Board of Studies in Physics, and the first honorary secretary of the Institute of Physics. He was elected a Fellow of the Royal Society in 1911. He served a term as President of the Röntgen Society; and also of the Faraday Society. At the time of his death he was one of the editors of *The Philosophical Magazine*.

Herbert Henry Woollard was born in Victoria, Australia in 1889. He studied medicine at the University of Melbourne, qualifying at the early age of 21. He served with the Australian forces in the war, attaining to the rank of Lieutenant-Colonel in the Australian Army Medical Service. After demobilization he began a course of study at University College, London, where he came under the influence of Elliot-Smith, and determined to take up the study of Anatomy as a scientific career. In 1923, he was appointed Assistant Professor of Anatomy. From 1928 to 1930 he held the Chair of Anatomy and Histology in the University of Adelaide. He returned to England to become Professor of Anatomy at St Bartholomew's Hospital Medical College. Finally he succeeded Elliot-Smith in the Chair of Anatomy at University College.

Woollard was a brilliant worker, full of energy and eager to suggest and carry out new ideas. Anatomy was to be far more than a descriptive science: it was to be linked with experiment. His researches dealt especially with the problems of histology, and he laid stress on the need for their consideration in conjunction with those of anatomy.

His technique was of a high order. He perfected certain methods of staining, and was able to advance by their means the general problem of innervation. He used his own body for experiments, which were often far from painless. He made a great number of important observations. He was able, for example, to identify specific types of nerve terminal subserving touch and temperature.

He was elected a Fellow in 1938. He was a fine teacher, researcher and administrator. Anatomical science suffers severely by his death when many more years of good work might have been expected from him.

Arthur Smithells (1860–1939) was in his early days a student at Glasgow University and attended some lectures by Kelvin. At the age of 18, having acquired a passion for science and especially for chemistry, he entered Owens College, Manchester, where he was a pupil of Henry Roscoe. In 1882, he graduated B.Sc. of London University. He then went to Germany and studied in the first place under von Baeyer in Munich and afterwards under Bunsen in Heidelberg. In 1883, he lectured on chemistry at Owens College and in 1885 he succeeded Thorpe in the Professorship of Chemistry at Yorkshire College, afterwards the University of Leeds. He resigned his Chair in 1923, and went to London to take the Directorship of the Salters' Institute of Industrial Chemistry. He was elected a Fellow in 1901.

Smithells when a young man gave proof of great ability as an experimenter; his work on "Flame" is well known and his beautiful illustrations are often repeated. But his main contribution to science has been elsewhere. His love for chemistry was not satisfied with the growth of chemical knowledge: he had a vision of the important part that science could play in the work and the social life of men, in their doings and in their thoughts. Chemistry was not merely a subject for academic study, nor was it only a tool of industry: it was both these and it was also a link between them, and in their combination it could be a lively force. As he held these views enthusiastically and had remarkable powers of exposition and of organization, he was able to assist notably in their realization. The University of Leeds with its considerable chemical school, its technical departments of fuel, leather, dyeing and textiles, all designed on broad lines, is greatly indebted to him for its success and for its true University character.

HENRY BALFOUR (1863–1939) was educated at Charterhouse and at Trinity College, Oxford, where in 1885 he took honours in the final school of animal morphology. A year previously the University had accepted the collection of ethnological and archaeological specimens made by General Pitt Rivers. Prof. H. N. Moseley, in whose charge the Pitt Rivers Museum had been placed, invited Balfour to assist him in its arrangement. He worked under Moseley until the latter's death in 1891, when he himself became Director of the Museum. In subsequent years the Museum grew in size and richness under his care.

The main principle in Balfour's work was in the first instance that which Pitt Rivers followed, and sought to illustrate by his collections and the method of their display. The evolution of mankind could be followed by examining the evolution of his tools, weapons and other objects which he used. In order to show this it was necessary both to collect and to arrange with a definite purpose and in an orderly fashion. No one could have been better suited than Balfour to develop this conception and to extend it. He had received a classical education, he was zoologist, field naturalist and artist all in one. He travelled widely in his search for evidence. The use which he made of his acquisitions established his own fame and that of the Pitt Rivers Museum.

He published many essays on such subjects as flint instruments, the crossbow, the fire-piston, kite-fishing, thorn-lined traps and many others of like nature; always arranging his material to show its evolutionary lesson and its contribution to ethnology.

ARTHUR PHILEMON COLEMAN was born at Lachute, Quebec, in 1852. He studied at the Victoria University at Cobourg in Canada and at the University of Breslau. He became Professor of Geology at his old University in 1881 and at the University of Toronto in 1890. He retired in 1922.

Coleman belonged to a type of public servant to whom the British Dominions are greatly in debt: men well versed in the knowledge of their day, and possessed of a physical vigour, a breadth of mind, and an adaptability which enabled them to attack the problems of wide territories, only half explored. The ordered development of the new countries has been made possible by their accurate and comprehensive observations and inferences.

Coleman was an explorer as well as geologist, and it needed much laborious travelling to map the dispositions of the strata in the Canadian wilderness. He is distinguished for his description of the great metal ore-field of Sudbury, and its place in the complicated series of the pre-Cambrian strata. He was interested in the history of past ice ages, and travelled widely in search of evidence. He published a well-known book on the subject. One of his water-colour sketches is probably the earliest picture of Mystery Mountain (Mt Waddington) in the Coast Range of British Columbia.

Coleman was elected a Fellow in 1910.

EDMUND BEECHER WILSON (1856–1939) was elected a Foreign Member of the Royal Society in 1921. He was one of the foremost zoologists in the United States. He began his academic studies at Yale University, and continued them at Johns Hopkins, Cambridge, Leipzig and Naples. He was in succession a lecturer at the Massachusetts Institute of Technology, Professor at Bryn Mawr College, and Director of the Zoological Laboratories at Columbia University. He was the Professor of Zoology at Columbia from 1891 until his resignation in 1928.

Prof. Wilson was particularly interested in all questions of heredity. He introduced into biology the conception of "cell-lineage". He wrote a number of books on microscopical anatomy, embryology, zoology and kindred subjects. His work gave him an international reputation, and he was a member of learned societies all over the world and an honorary graduate of many Universities. He was the Croonian Lecturer of the Royal Society in 1913.

HARRY MEDFORTH DAWSON died on 9 March at the age of 63. All his life, with the exception of three years of study in Germany, was spent at Leeds. He was a scholar at the Leeds Modern High School, and afterwards he was in turn student, demonstrator, lecturer and professor of chemistry in the University.

In the last year of his course of study in the University he was engaged on research work in collaboration with Prof. Smithells, whom also we have lost during the past year. He won an 1851 Exhibition scholarship and spent three years at Berlin, Leipzig and Giessen. He was one of a group of able young men who represented the growth in England, at that time, of a new spirit of research, and like many of his chemical contemporaries sought to develop it in Germany.

Dawson's work was centred round the mechanics of solutions. He was an enthusiastic student, to whom no pains were too great for the purpose of accuracy and fullness of proof. He did a fine piece of work in his establishment of the multiple theory of catalysis.

Dawson was essentially a laboratory worker. He inspired a number of student collaborators and he was greatly interested in the researches of others in fields apart from his own. He was for some time the secretary of the Priestley Club with a membership which included many outside the University. His own kindly nature enabled him to maintain with success the friendly informality of the Club. He was elected to the Royal Society in 1933.

Sir HENRY ORAM who died on 5 May of this year, at the age of 81, was a very distinguished naval engineer. After years of great promise at the Engineering College at Keyham and the Royal Naval College at Greenwich and two years at sea he was appointed at the Admiralty in 1884. For thirty-three years he took a prominent part in those radical transformations of the machinery of the Navy which were necessitated by modern science and inventiveness. Water-tube boilers were introduced while he was deputy under the Engineer-in-Chief, Sir John Durston. He took a prominent part in the change from reciprocating engines to turbines. It was largely due to his advocacy and skill that turbines were successfully fitted in H.M.S. *Amethyst* and subsequently in the first Dreadnought. When oil came to the front as a fuel, and a Royal Commission under Lord Fisher reported on oil fuel and oil engines, Oram was warmly praised by the Commission who "without his aid, could not have so confidently expressed their unanimous conviction of the vital necessity for fighting ships of oil fuel and oil engines". Under his directorship, Diesel-type machinery was adapted for use in submarines, a work requiring the highest skill.

Oram was elected a Fellow in 1912 in recognition of the fact that his labours led not only to engineering achievements but also to the advancement of science. Those who worked with him at the Admiralty testify to a personality which ensured their grateful and devoted service.

Frank Watson Dyson was born in 1868, and was educated at Bradford Grammar School and Cambridge. He was second wrangler in the Mathematical Tripos in 1889 and was elected to a fellowship in Trinity College. In 1894, he was appointed chief assistant at the Greenwich Observatory. He became Astronomer-Royal for Scotland in 1905 and returned to Greenwich as Astronomer-Royal in 1910, retiring in 1933. He was knighted in 1915.

Dyson's first important work was a completely new reduction of the Groombridge observations of circumpolar stars. The results were so accurate and reliable that Eddington was able to use them in his extensions of Kapteyn's principle of star-streaming. When he returned to Greenwich he took in hand the collection of accurate information respecting the stars comprised in the zone extending from 65° N. to the pole, this being the zone allotted to Greenwich by international agreement. Proper motions of the stars were deduced from differential measures of photographs taken at sufficient intervals.

Dyson took a great interest in the problems that could be solved by observations taken during total eclipses of the sun. He took part in several expeditions: he was always remarkably lucky in his weather. He had much to do with the famous expeditions that went to Principe and Brazil with the intention of testing the Einstein theory.

Dyson was universally popular among scientists at home and abroad, and was therefore able to play a prominent part in the formation of the International Astronomical Union.

The success of Dyson's career as an astronomer was due not only to his mathematical and experimental abilities and his organizing capacity, but also to personal qualities which made friends and helpers of all who had to work with him and under him.

John Mellanby, born in 1878, was one of the first students of the biochemical school founded by Gowland Hopkins in Cambridge. After taking his degree he went in 1900 to the research laboratories which Burroughs and Welcome had recently founded at Brockwell Hall. Inspired no doubt by the teaching he had received as an undergraduate he began a research on the properties of serum proteins and their relation to antitoxins. After studying medicine at Manchester he took his M.D. at Cambridge in 1907. For a short time he continued his researches at the Cambridge laboratory, being particularly interested in the phenomenon of clotting in blood and milk.

In 1909 Mellanby left Cambridge for St Thomas's Hospital, where he used the clotting-time of milk to determine the quantity of active trypsin in pancreatic juice, and thus to observe the formation of trypsin from trypsinogen. From the properties of trypsin he went on to the mechanism of its secretion and it is perhaps his work on this and on the purification of secretin which, together with his earlier investigations of clotting, form his most prominent contributions to knowledge. In 1937 Mellanby became Professor of Physiology at Oxford where he continued his highly important researches.

Mellanby was elected to our Fellowship in 1929, and was at the time of his death a valued member of our Council. He was also a member of the Medical Research Council. He was not only a fine investigator but also a man of wide views who was influential in asserting and strengthening the ties between physiology and medicine. In this work he was helped by the respect and affection which his kindly and earnest nature inspired in his fellow-workers.

ALFRED HARKER (1859–1939) graduated at Cambridge as eighth wrangler in 1882. He was elected to a Fellowship at St John's College in 1885 and was appointed a demonstrator in geology under McKenny Hughes. In 1918 he was elected to a special readership in petrology.

Throughout his long and active life Harker devoted himself, in the main, to the relations between the characteristics of igneous rocks and the controlling influence of tectonic environment. His first papers dealt with the cleavage properties of slates; his mathematics were here of great assistance to him. This work led him to study the petrology of the igneous rocks associated with the Cambrian system of Caernarvonshire. It is described in the Sedgwick Prize Essay of 1889. In 1889–93 he was engaged with J. E. Marr on parallel problems in the Lake District. In 1895 and succeeding years he was attached to the Geological Survey of Scotland. He devoted himself to the stratigraphy and petrology of several of the Western Isles and completed a successful survey of the mountainous groups of central Skye

In 1909 the wide knowledge which he had by that time acquired was embodied in his *Natural History of Igneous Rocks*, an outstanding book which has been of fundamental importance in the growth of petrologic science. When advanced in years he continued to write with power, and his *Metamorphism* published in 1932 is a fine piece of work. He completed the revision of the MS. for a second edition shortly before his death.

Harker was elected to our Society in 1902. He was awarded a Royal Medal in 1935; and received many other recognitions from other Societies, and from Universities. His influence on petrology at Cambridge and throughout the world was very great: the Harker Collection of some 40,000 specimens of rock slices will ever serve as a monument of his work. Many senior students found inspiration in his lectures and remember him with affection and respect.

SIGMUND FREUD was born in 1856 in Moravia. He took up the study of medicine, and while still in the twenties went to Paris to study psychiatry under Charcot. At the age of thirty-one he returned to Vienna where he lived until the recent advance of Germany into Austria forced him to take refuge in England.

Freud was the originator of a form of research to which the term psychoanalysis is now applied. It has been described as the empirical study of *unconscious mental processes*. Considering the elusive character of work in this new field, and the extraordinary and unconventional conclusions to which it has led under the guidance of Freud and an enthusiastic body of collaborators, it is not surprising that Freud's ideas have provoked both bitter opposition and warm approval. It may well be too early to estimate correctly the value of this novel work, but certainly it has provoked much thought and experiment, and its supporters maintain that it has done much to explain and alleviate human distress.

Britain was glad to welcome the old man when he came as a refugee to spend his last years here. The Society elected him a Foreign Member in 1936. When he came to this country the opportunity was taken to obtain his signature in the Charter-book of the Society. In deference to his ill-health and in recognition of his great distinction, the Officers of the Society took the book to his home for signature, a step which had never been taken before.

ROBERT SCOTT TROUP was born in 1874, and was educated at the University of Aberdeen and at Coopers Hill College. He joined the Indian Forestry Service in 1897, and was Deputy and Assistant Conservator of Forests in Burma until 1905. He was then appointed to the Forest Research Institute at Dehra Dun. From 1915–19 he was Assistant Inspector General of Forests to the Government of India. He left India to become Professor of Forestry at Oxford.

The care of forests has become of recognized importance in the economy of the Empire, and men such as Troup have been of the greatest value in the development of the necessary knowledge and technique. The forestry of Burma was already famous when he began his apprenticeship there. When he went to India with a reputation already considerable he entered upon a term of service which proved to be of the highest value to the forests of India and to forestry in general. He wrote fully and well of his experiences, so that his books, particularly *The Silviculture of Indian Trees*, are widely studied, and accepted as authoritative by the Indian and Burmese staffs. He wrote also on forestry in other countries and only a year ago he published a very interesting book on *Forestry and State Control*.

Troup was elected a Fellow in 1926. He did much for the development of Forestry science, and because of his knowledge and his devotion to his work, he served the nation well.

In October death removed from our Foreign Membership, HARVEY CUSHING, a man of great professional accomplishment, and of singular personal charm. Six months ago his 70th birthday had brought together to Yale a remarkable gathering of his old pupils and others, to testify to him their admiration and affection. The spirit prompting the gathering is indicated by the circumstance that one of those present crossed and recrossed the Atlantic though able to stay the one day of the celebration only.

Dr Harvey Cushing will be remembered as a great Surgeon. He was great in surgery not only by reason of his successes as a skilled operator, and of his experience in a field he had practically made his own. He was great also by reason of the new approaches he brought with him to his subject. With him the neuro-surgeon was not limited to being an executant at the behest of the physician. Cushing was, where the nervous system was concerned, his own physician and combined within himself the skill and knowledge of the physician as well as of the surgeon. He attracted pupils and followers from many parts of the world. The way in which he proceeded with a case, the details gone into, the deliberateness of the steps, the patient protractedness, as if time were but for slaves, led sometimes to the remark that his work was like a physiological experiment carried out on man. His surgery had notable repercussions upon physiology. He ascertained, by interrogation, the human being's experience when the surface of the brain is electrically stimulated. His surgical dealings with and enquiries into the pituitary body greatly contributed to knowledge of that gland, and its inter-relations with an adjoining region at the base of the brain, and further of the ties between it and the nervous regulation of the viscera. He found therefore the influence of that forward-lying part of the brain to be more full and direct upon the viscera than had been previously ascertained or thought likely. Perhaps Cushing's greatest contribution to the study of the brain regarded tumours affecting it, where his experience was unique in its extent.

In the war of 1914–18 Cushing served as a military surgeon first in the British Army and later in the American. Three years ago he published an ample volume of extracts from his Journal kept during the War years. But his chief literary production was his *Life* of his teacher and older friend, William Osler, the physician. Theirs was a fraternal friendship. Cushing was a most lovable as well as gifted character.

Sir WILLIAM JACKSON POPE, who died on 17 October 1939, aged 69 years, was an outstanding figure in the world of chemistry.

A pupil of H. E. Armstrong, he occupied successively the positions of head of the chemical department at the Goldsmiths' Institute at New Cross, and at the Manchester Municipal Technical College, and was in 1908 elected to the Chair of Chemistry at Cambridge.

His scientific work was chiefly concerned with the molecular configuration of organic compounds and he extended our knowledge of this branch of chemistry in many directions. His early achievements in resolving compounds of nitrogen, sulphur and selenium into optical antipodes form a landmark in the history of stereochemistry and opened a wide field of investigation. His work on methyl*cyclo*hexylidene acetic acid was no less notable. The conception of this compound, its synthesis (with Perkin and Wallach) and its resolution into enantiomorphous forms was pioneer work, revealing the existence of classes of compounds whose dissymmetry was better referred to the configuration of the molecule as a whole than to the presence of an asymmetric atom.

The exceptional breadth and accuracy of his chemical knowledge and his strong practical sense enabled him to render conspicuous service to the Government during the last war and in 1919 he was made a K.B.E.

Endowed with a strong personality and unusual administrative power he took a prominent part in chemical affairs. He served as President of the Chemical Society and of the Society of Chemical Industry. He was the first chairman of the Federal Council for Chemistry. In 1922 he was chosen to preside over the International Union for Pure and Applied Chemistry in the formation of which he had been intimately concerned. He was for many years President of the Solvay Chemical Conferences at Brussels, a position for which he was exceptionally qualified by his fluent command of French and German.

He was elected a Fellow of our Society in 1902 and in 1914 received the Davy Medal. Numerous honours were conferred on him; he was a foreign member of many chemical societies and a Corresponding Member of the Académie des Sciences.

Ralph Allen Sampson was Astronomer-Royal for Scotland from 1910 to 1937. Previously he had been Professor of Mathematics in the University of Durham. His greatest work was a study of the four Galilean satellites of Jupiter, for which he received the Royal Astronomical Society's Gold Medal in 1928. This was a comparison of the accumulated observational data with the intricate dynamical theory of the motions. A memoir on the Sun which he published in 1892 is remarkable as being the first application of the conception of radiative equilibrium to the structure of the stars; in this he was much ahead of his time, but progress was then limited by the backward state of the physical theory of radiation. At Edinburgh he took up especially the practical determination of the colour temperatures of stars.

Sampson took great interest in the optical problems of telescope construction and in the accurate measurement of time. He was deeply versed in the history of astronomy and mathematics.

He was born in County Cork in 1866; he was elected a Fellow in 1903.

The tragic happenings of these times have necessarily had their effect on the activities of the Society. On the outbreak of War, the offices were moved—as had been decided sometime beforehand—to Trinity College, Cambridge, and I take this opportunity of expressing the gratitude of the Society for the hospitality which we have found there. We could not have wished, if we were to be in exile, any greater happiness than to be housed in the College of Isaac Newton and many another of our Fellows, past and present. Many of our irreplaceable possessions were removed to places of safety.

The meetings for the reading of papers have been suspended temporarily, but their early resumption is possible, and will certainly take place if circumstances allow. The publication of papers has not been interrupted though it has seemed well to place some limits on their length. Most of the other activities of the Society, including the administration of funds for research, are proceeding as usual.

The Council's Report deals with the business of the Society during the past year, and the second volume of Notes and Records tells of many other matters of interest. I am thereby relieved of the necessity of referring in this Address to several subjects already considered. I propose to say a few words on the general position of the Royal Society at this special time. But first I would speak of the debt which the Society owes to its retiring Treasurer, Sir Henry Lyons. We owe to him the complete and valuable reform of our finances carried out during years when the monetary transactions and responsibilities of the Society have been growing at a great rate; he has made welcome improvements in the conduct of our business; our house and rooms are much the better for his care: nor can we forget the successful introduction and maintenance of "Notes and Records" to which I have already referred. In very many other ways we are his debtors. The Society parts reluctantly with the Treasurer who has so splendidly filled his office for the past ten years. Perhaps his effectiveness does not disappear with the tenure of his office, for as long as he is with us his critical yet friendly eye will make it impossible for any one to be careless in the Society's business.

A very large number of our Fellows are serving in different capacities for the prosecution of the War. Twenty-five years ago the War, which to so many of us seems to have been resumed after a very short interval, taught the world the importance of science and its application, and that not merely in the war itself but in many subsidiary activities and indeed by example and by inference in all human undertakings. The lesson has not been forgotten in the years that have intervened, and the present outbreak finds all our Defence Services far ahead of 1914 in the employment of science and scientific men. For this we must be profoundly thankful; for it is clear now that any nation which fell behind in this respect by even a few years of study and application would quickly be overwhelmed. It is early to form a complete estimate of the effectiveness in the present war of the attention that has been given to science in the last twenty years, but there is no little promise in what we are permitted to know of what is going on. There is evidence that the application of science in all sections of war-activity, land, sea and air, are proving their value and more than justify all that has been spent upon them.

There is indeed a widespread recognition of the general effectiveness of science. The ways of using science and scientific men are being slowly discovered. But the process is slow. It would, I think, be hastened, if certain fundamental truths were generally known and recognized. I venture to state them in the form of a few propositions:

1. Science, that is to say, the knowledge of Nature, is of fundamental importance to the successful prosecution of any enterprise.

For example, a nation is obliged to make all possible use of science in preparation for war, whether aggressive or defensive: and, again by way of example, in the maintenance of public health and social welfare. Of course, science is not alone in being a necessity in either case.

2. Science is of general application. There are not *one* science of

chemistry, another of electricity, another of medicine and so on: there are not even distinct sciences of peace and war. There is only one natural world, and there is only one knowledge of it.

Experience shows that an advance in knowledge or technique or skill in any direction may be based on some item of knowledge acquired in a far distant field of research. For that reason, it is necessary to resist strongly a natural tendency for those who study science or apply it, to separate into groups without mutual communication.

3. Fruitful inventions are always due to a combination of knowledge and of experience on the spot. Unless the man with knowledge is present at the place and the time when some experience reveals the problem to be solved he misses the fertilizing suggestion. Neither can the mastering idea suggest itself to the man who has the experience only but no knowledge by which to read the lesson that the experience teaches. The man with knowledge may be a temporary or special introduction, or, which is much better, he may be the man who meets with the experience.

4. There are difficulties peculiar to the application of science to war purposes. While the war proceeds scientists as a body are anxious to put all their knowledge at the service of their country: but when the time comes they are anxious to get away to their work on pure science or the applications of science to the problems of peace. Government may preserve and most fortunately has preserved a nucleus of able scientific effort during the last 20 years of peace, so that a certain connexion is maintained between these particular applications and the general body of science: but from the very nature of their respective occupations, and on account of a certain secrecy which one of the two bodies is forced to maintain, the connexion is not always strong. It can easily happen that the solution of a particular difficulty in the war service may lie in some piece of knowledge far away from the immediate science of the enterprise and unknown to those who need it.

I believe that the four statements which I have just made and briefly amplified are true, and must be acknowledged to be true by all careful observers. That being the case, it is of the utmost importance to move in the direction in which they point. We must ourselves be interested in any attempt to do so. We must wish to see that the vast and growing body of natural knowledge is most effectively employed in the service of the nation.

The tremendous use of science in the present war compels us to think about the method of its use. Many suggestions have already been made: as, for example, that a Ministry of Science should be formed immediately.

In my opinion there is no solution here. A ministry would be too formal and rigid at any rate for immediate needs; the most successful ways of using knowledge are personal and elastic. We must not attempt too much at once. We might be content if we could in some way bring Science as a whole into close relation with Government as a whole, if we could attach a central authority of science to the central authority of the country. The immediate application of science in any department of the country's business should be made from within the department, not from without, and we have already a number of instances in which this principle is followed. The Department of Scientific and Industrial Research, which we are proud to think of as an original device of the Society, correlates many branches of industry with science: it is entrusted with the administration of large funds, and has had great successes. Similarly, the Medical Research Council administers funds and encourages research in the interests of the health of the people, and again the Agricultural Research Council has its own field of action. Each department of the Defence forces takes care of its own applications of science. This is satisfactory so far as it goes. The need is rather for means whereby the Government, in its care for the whole sweep of the country's business, can rely on and make use of the whole range of scientific knowledge.

It would seem that our Society itself is a body which is not used as it might be, though, as I have said already, a large number of our Fellows are individually taking part in the application of natural knowledge. Within its ranks are to be found men with knowledge of every form of natural science. It would be well if by way of some small group of Fellows, selected for the purpose, it were consulted without hesitation whenever the need arose, and if it were kept so well informed that it might foresee occasions and needs. If that could be achieved, a forward step would be made, which in due time would be followed by others. It would be a very proper step to take, being a continuation and extension of a process which already exists. To use a modern term, science is irresistibly making its way into general use by a "peaceful penetration", which in the end is far more effective than a violent movement; all the more so because science is not out to fight present methods of rule and economy but rather to strengthen with new knowledge of facts and principles. We have often proved in this country the practical value of making use of existing institutions rather than founding new ones, when the old are full of vigour and can carry new grafts without strain. That is why the Royal Society, as a body of high consultative value, is ideally fitted to form the next link between knowledge and practice.

There are two subsidiary consequences of the present position of science which need to be remembered. We have not long passed the stage, if indeed we have quite gone past it, when our knowledge of Nature is used as a reference library is used. The knowledge is there, on the shelves, to be taken down when anyone wants information on a particular point and happens to remember where possibly it may be obtained.

Anyone who wants to use a library effectively must already have some knowledge of the same nature as that which he hopes to find there. If not, he does not know where or how to look; nor can he grasp fully what he finds, even if he happens to hit upon the right book. For a parallel reason, it is most important that a general knowledge of science should be diffused among the people, and especially among those to whom it falls to guide and govern.

A second consequence is that the supply of men capable of the study and interpretation of the natural world must be made continuous. An instance, special to the moment, is the present policy of reserving able men in their final year at Universities and technical institutions so that they are not absorbed too early into the fighting services nor into inappropriate branches thereof. This is necessary; but it is not all that is necessary. National interests demand that the water shall not be diverted higher up the stream.

And it may be well to add here, that the care for those who can in due course be expected to supply the knowledge of Nature and to assist in its use, is not the nation's only care. It would be absurd to overlook other knowledge: I am but limiting myself to matters with which the Society is especially concerned.

Let me now turn to a question of pure science. When the new methods of X-ray analysis were first introduced a quarter of a century ago there was naturally no clear realization of the extent and character of the fields of research in which they would eventually be employed. Certain applications were found for them at once, but the years have brought wide and unexpected developments. Some of these are gathering themselves together, and, in conjunction with other methods of physics and chemistry, begin to form what might well be called a new branch of science.

In the early phase of the X-ray crystal studies the object of interest was the perfect crystal; in the later phase attention is directed towards the departures from perfection, which turn out to be of the greatest interest and importance.

It will be remembered that the method of analysis of the structure of matter by means of X-rays was based upon a suggestion made by Dr Laue.

If the atoms in a crystal were in regular array, the passage of ether waves through the crystal should be accompanied by diffraction effects provided that the lengths of the waves were of the same order of magnitude as the spacings of the atoms. In the case of the ether waves emitted by an X-ray bulb—if indeed the X-rays were ether waves, of which there was some doubt at the time—there were reasons to suppose that the wave-lengths were of the magnitude required. The experiment was made, and was successful.

The diffraction effects provided means whereby the crystalline arrangements could be calculated. The researches of the first few years of X-ray analysis were therefore concerned with the character of the crystalline arrangement in a number of the simpler cases. As confidence grew and skill increased more difficult structures were attacked, and indeed it has been very surprising to find what complicated structures can be unravelled. One helpful circumstance has been the existence of families of substances, since the progressive differences in the members of a family gave rise to corresponding changes in the diffraction patterns. Thus, for example, the large family of silicates was examined and the connexions between composition and diffraction effects, and again between the latter and structure, were observed and compared. Certain simplicities and uniformities then appeared and it became possible to put in order a mass of details which had not previously appeared to have any relation with each other.

It was not long before the results in the field of research became so numerous that books of no small size were required to contain them. In thousands of cases the dimensions of the unit cell of the crystal were determined, and at least the space-group or character of the arrangement of the atoms within the cell. In a number of these cases the exact relative positions of the atoms could be found, though this additional task has often been formidable. Work of this kind continues, and rightly so, to be the occupation of many investigators. All such work belongs to the first phase to which I have referred.

The application of the X-ray methods has for some time been entering on a second phase. It now deals with a natural phenomenon differing entirely from that which was the first to be examined. The earlier work was concerned with the arrangement of the atoms in a perfect crystal, that is to say a body in which the mutual forces are balanced, and the arrangement is complete. Thermal movements may still be there, but the average dispositions of the atoms are settled, and are uniform throughout the body of the crystal.

It is doubtful whether there is such a thing as a perfect crystal large enough to be handled; perhaps the crystals of diamond and graphite are nearest to perfection. In almost all cases there are deviations from complete uniformity. A crystal that has every appearance of being perfect may consist of an assemblage of minute crystallites, more nearly perfect individually but lacking uniformity of orientation to a greater or less degree. Other bodies that do not appear to be crystalline at all may consist of crystallites oriented so irregularly that the bodies seem to be isotropic. The crystallites may vary in size as well as in relative orientation. Two or more crystal forms may be present in the same body, so mixed together that only the X-ray methods can make any attempt to disentangle them. Sometimes one greater lattice overrides in a more or less regular fashion a smaller lattice as a pattern of ploughed fields may override a pattern of furrows. Also a lattice may be distorted by strain. In a substance in the liquid state there may be associations and partial arrangements sufficient to show X-ray diffraction.

Moreover, arrangements and dimensions may vary with time, both in solids and liquids; some forms or extents of arrangement may even disappear, new forms may appear at the expense of the old. Perfection of crystalline arrangement is a goal which is never achieved. And in fact the processes of the world are based upon such deviations from perfection, and their continuous modification. If all arrangements of atoms were complete, there would be nothing left but stagnation and the peace of death.

It will be seen therefore that a new field of research of extraordinary interest is being opened up. It is in this field that the physicist and chemist and indeed scientists of every persuasion must look for explanations of many of the properties of their materials such as their relations to magnetism and electricity, thermal conductivity, tensile strength, various surface effects and so on: as also the time-changes in these properties, such as the creep to which the engineer has to give so much attention. Properties such as these have been called by Orowan and others the "sensitive" properties since they depend on the particular state of a body, which state in turn depends largely on the conditions of the new field of which I am speaking. The biologist finds interest in it, because the life processes seem to involve the relations between large aggregates, molecules or assemblages of molecules, with one another or with media in which they are imbedded. Somewhere in this field life and matter are first found in association.

It may seem unreasonable to expect help in the resolution of such complications from the X-ray diffraction effects. It is to be remembered, however, that the X-ray photographs are very rich in information. The gratings are three-dimensional and may be examined from a variety of aspects; each photograph or spectrometer record is a two-dimensional diagram of positions and intensities of diffracted spots. A light spectrum of the ordinary kind is uni-dimensional only. The photographs vary in definition, being less easy to read as they deal with more complicated cases; but the technique is rapidly improving.

The earliest of these phenomena of the larger field was the so-called mosaic effect. A crystal of rocksalt, for example, is a mass of small crystals, each of which approaches to regularity far more completely than the whole. The separate crystallites are not in perfect alignment with each other. Hence arose one of the perplexities of the early days. It was extraordinary to find that the less perfect crystal reflected the X-rays in greater intensity than the more perfect and that the reflections from the face of a crystal, quartz, for example, might be increased if the surface was roughened. The puzzle is solved if we remember that, as Darwin and others pointed out, one crystallite may screen another. If the orientations of the two are exactly the same, and they are set at the proper angle for reflecting the incident X-rays the first crystallite will partly absorb the rays in producing its own reflection and the lower will not have its full opportunity. But if, as is usual, the crystal is moved through small angles about the above setting so as to give every crystallite its chance—provided that the X-rays can penetrate the crystal so far as to reach it—and if the second is in the right orientation for reflection when the first is not, then the incident rays get through the first and are reflected by the second; the integration of all effects therefore gives a larger total when the crystallites are not parallel. This mosaic character is very common, even in crystals of the purest material. There are indications, as remarked by Goetz and others, that in some cases at least it is the state of greater equilibrium. Thus W. A. Wood shows that crystals of copper and other metals of extreme purity are reduced by cold working to crystallites in more or less complete disarray. The substances do not then return to their former state, though they can be taken some way towards it by moderate heating. To restore them to their original state it would be necessary to begin over again from the melt. A similar effect was shown by Ewald to occur in the case of rocksalt. It may also be significant that of the two forms of diamond examined by Robertson and Fox, that which is the more transparent to infra-red and ultra-violet and may therefore be taken to be in greater internal equilibrium, is also the one in which there is some mosaic character.

If this form of disintegration of a larger crystal into crystallites is really due to the release of energy, it might be expected to proceed until the process was complete and the substance became amorphous. Wood shows that it does not proceed indefinitely and that the copper crystallites have an average linear dimension of about 700 A; for silver the figure is 800, for nickel 1200, and so on. The disintegration ceases at a certain point. The metals were of extreme purity. Unless it appears that the mere trace of impurity governs the effect, it must be supposed that the disposition to form aggregates of definite magnitude is present in the copper atoms themselves.

The presence of crystallites of a definite pattern, their dimensions, their preference for any particular orientation in relation to the body which contains them and their proportional amounts are all determined by the X-ray photographs. It is to be remembered that the diffraction spectra of a compound of different crystallites do not modify nor disturb each other's evidence. The data on which calculations are based are the forms and intensities of the spots and lines in the X-ray photography. The calculations are often difficult and lengthy because so many factors have to be taken into consideration; in fact there is plenty of evidence, and the most troublesome part of the business is its interpretation.

It will be readily understood that such measurements as these can be of great assistance in the study of metals. It is possible to examine in a new and most effective way the phase diagrams of the metallurgist, and the various effects of composition, temperature and time, and this not only for binary alloys but also for ternary and still more complicated mixtures. With these powers in hand, and with the remarkable accuracy of the modern X-ray spectrometer which can show minute changes of form due to temperature or an admixture of foreign atoms and can show also any variations in the extent of order and disorder in the atomic arrangements, it is not surprising that theoretical metallurgy has acquired a new life and that practical metallurgy begins to gain thereby.

Since the X-ray methods can do so much to discover the composition of molecules, even very large molecules such as the phthalocyanines (Robertson) or the sterols (Bernal, Crowfoot and others), and also to determine the arrangement of the molecules in crystalline aggregates and yet further to find any preferred orientations that there may be, the methods can be fitly used for the examination of fibrous materials. In cotton, silk, rubber and many other substances there are long chain molecules which are linked together into crystallites having preferred orientations along the length of the fibre. So also nerve, muscle, horn and such like contain proteins and keratins in fibrous forms. It is even possible, as Bernal has shown, to find some details of the composition, structure and arrangement of immense molecules such as those of a virus. Very interesting papers on these subjects have been communicated to our Society, some of them during the present year.

It is to be observed that the extension of the X-ray methods to these larger scale problems is greatly facilitated by extrapolation from simpler observations. In the case of organic substances the modes of assemblage into characteristic combinations of atoms, such as the association of carbon atoms in the benzene ring, are governed by rules which are so closely followed in the simpler cases that they can be assumed to hold in the more complicated. These rules relate to the distance from atom to atom in various cases, and the mutual orientations of atoms. In this way very important suggestions have been made in respect to the construction of the more difficult assemblages.

It is to be remembered, of course, that there are other physical methods of observation which contribute to the understanding of the complex substances of Nature, which are all the more efficient now that the X-ray methods are able to make their characteristic contribution. First of all comes chemical science, the power of which is so obvious and well known that I need not do more than refer to it. Optical properties have been found to be extremely useful. So also the magnetic properties are found to be closely related to structure. Thus, for example, the disposition of a molecule containing benzene rings can often be predicted from diamagnetic measurements.

The sum total of these powers, some new some old, all reinforcing each other in a common advance, is so great that, as I have already said, a new field of enquiry of first rate importance has been well entered. That which the eye can see is one thing: that which the microscope reveals is another. Far beyond any vision is the individual atom and the atomic nucleus, of which so much has been discovered in recent years. But a vast range of magnitudes, lying approximately between 10 and 10,000 A has never been accessible to the direct attack which the ranges on either side of it have experienced. Within this range lie all the processes which are concerned in the building of living substances, animal and vegetable, and in the changes of growth and decay. In this range, lie also elements that are the origin of the properties of our materials, alloys, glasses, fibrous substances of all kinds, and here take place transformations which change these properties some of them rapid especially when urged by heat, some so slowly that centuries must pass before they become visible or effective. The recent advance into this new field is only the preliminary to what is sure to follow. We have before us an enquiry of supreme interest.

Awards of Medals, 1939

The COPLEY MEDAL has been awarded to Professor THOMAS HUNT MORGAN.

In those branches of biology which are called Botany and Zoology the most important advance during the present century has been the development of Genetics. The establishment of definite laws of heredity, and the discovery of the mechanism, the gene, by which hereditary qualities are carried on from generation to generation, has revolutionized our outlook on the function of the nucleus of the cell and of the chromosomes it contains; it has enabled us to understand the significance of the maturation of the germ cells and of fertilization as they occur in higher animals and plants, and thus led to a very rapid development of nuclear cytology. The theory of the gene has given us a new outlook on the determination of the development of an animal or plant. But nowhere has genetics produced greater changes than in our attitude to evolution. The observation that mutations arise *de novo* at a definite rate, that the number of different mutations occurring in a single species may be very large and that the mutations in allied species are essentially identical, have shown us for the first time the materials which are available as a basis for evolutionary change. The quantitative nature of genetics has made it possible to examine the effects which result after many generations from the establishment of a community by a few individuals of different hereditary composition, and to estimate the effect of a definite advantage attaching to one particular quality on the ultimate composition of a population. It has thus put the Theory of Natural Selection on a sound theoretical basis. Furthermore it has enabled us to observe indirectly the effects of natural selection in wild populations, and to plan experiments to determine its effects.

The practical applications of genetics are as important as its influence on theory. The whole of the breeding of many cultivated plants including maize is now firmly based, and the process of improvement immensely hastened by cytological examination. Genetics is already influencing animal breeding, and in its modern developments is throwing much light on the possibility of controlling hereditary diseases in man. Thus the influence of genetics is already very widespread, and it will become important even in fields in which it is now little appreciated.

In the development of genetics the work of the Morgan school has been paramount. It is to Morgan that we owe that exploitation of *Drosophila melanogaster* which is the basis of most modern developments. To him we owe the theory of the gene, which is fundamental, and the explanation of "crossing over" which forms the basis of the conception of the linear arrangement of the genes and the chromosomes. From these starting points all other work springs, and much of this work comes from Morgan himself, his associates and his students. Thus, although in the case of a man working for many years in the closest association with very able men, it is impossible to isolate his individual contributions, it is evident that Morgan has done more than any other man to establish genetics and thus to revolutionize our ideas in many different fields of work, in practical affairs as in pure theory.

A ROYAL MEDAL has been awarded to Professor PAUL ADRIEN MAURICE DIRAC.

Dirac's chief work has centred around the fundamental principles of modern theoretical physics. The new quantum mechanics was discovered by Heisenberg in 1925. Dirac at once realized the great importance of this discovery, started to work out its fuller implications and by a remarkable combination of originality, mathematical skill and uncanny instinct rapidly established himself as a great leader in this field. His earliest efforts were directed towards tidying up the intermediate field between that of the new quantum mechanics and the Newtonian. In this connexion he established the importance of the Poisson bracket expressions of the old mechanics. An early and very important paper was on the fundamental equations of quantum mechanics. This was a generalization of any earlier work in this field and for the purpose he introduced a new algebra, that of non-commutative numbers.

Probably his greatest achievement was that of ending the conflict between quantum mechanics and relativity mechanics by showing how to make the fundamental equations of quantum mechanics invariant under a Lorenz transformation, at any rate to a first approximation which has not yet been improved on. This led to a revision of the theory of the hydrogen atom which confirmed Sommerfeld's formula for the fine structures of spectrum lines and X-ray levels. This formula had been derived hitherto from a mixture of empirical results and theoretical guidance but had not been deduced from general fundamental principles. The idea of a quantized electron spin also fitted naturally into the new theory. The harmonization of the quantum and relativity mechanics also required the introduction of the strange conception of negative energy states (holes) and this is generally regarded as a prediction of the existence of the positive electron (positron) since discovered by Anderson.

His book *The Principles of Quantum Mechanics* is an important and original work, a comprehensive account of the subject by an acknowledged master in this field.

A ROYAL MEDAL has been awarded to Professor DAVID KEILIN.

Keilin's contributions to entomology extend over nearly thirty years. In the main they concern the higher Diptera: with critical ability and a great capacity for detailed observation he has demonstrated the very close correlation that exists between larval structure and habits in these insects. As the result of Keilin's work it has become possible to determine the habits of almost all such larvae from an examination of their mouthparts and pharyngeal ridges. Among his many papers those on carnivorous Anthomyiidae and on the remarkable life-cycle of the parasite of *Pollenia rudis* deserve especial mention.

Keilin's main contributions to biochemistry have been his studies on cytochrome, published in a series of papers from 1925 onwards. These have filled an important gap in our knowledge of cell respiration and, in particular, have shown how various mechanisms, the existence of which was already known, are related to one another through the linking mechanisms which Keilin himself has discovered. He has shown that cells contain a respiratory pigment, cytochrome, composed of at least four components related to haematin. One of these is in all probability the enzyme previously known as indophenoloxidase. The catalytic activity of this enzyme depends entirely on co-operation with the other three components of cytochrome. The complete cytochrome system forms within the cell a highly active catalytic mechanism which by utilizing molecular oxygen can easily oxidize hydrogen atoms of certain substrate molecules which have been activated by dehydrogenase systems. This shows that the enzyme component of cytochrome may be identified with the previously undiscovered oxygen-transporting enzyme of Warburg and his co-workers.

Other work of Keilin in this field has included the characterization of certain oxidizing enzymes which make use of molecular oxygen, and the preparation in a pure condition of the polyphenol oxidase of mushrooms. The latter has been shown to be a copper protein compound. Recently he has also isolated in a pure state copper protein compounds from blood corpuscles and liver (haemocuprein and hepatocuprein) which may be of biological importance though they are not apparently concerned in oxidase reactions. Keilin has thus provided us with an integrated picture of cellular respiration which, though it does not cover all respiratory mechanisms, is an énormous advance on the much less systematized knowledge that we had before his work was published.

The DAVY MEDAL has been awarded to Professor JAMES WILLIAM McBAIN.

McBain's claim to special recognition rests essentially on the circumstance that he created, and has led the development of, a new and important chapter of physical chemistry—the study of colloidal electrolytes. It was as the result of a long series of precision measurements on the electrical and thermodynamic properties of soap solutions that McBain originally defined this new class of materials, which combined in a special way the properties of colloids and electrolytes. The definition and constitutional theory proved to be the key to the orderly exploration, which thenceforth proceeded with continually growing impetus, of a large and fruitful field—incidentally one of considerable technical importance. The materials include soaps, nearly all modern synthetic detergents, a number of inorganic substances such as silicates and tellurates, as well as many dyes, proteins and biocolloids. The value from a physical point of view of their classification as colloidal electrolytes, and the general correctness of the micellar description of the thermodynamic and electrical properties which distinguish these materials, are to-day universally recognized.

In the continued investigation, in which numerous workers have contributed to the general development of the subject, McBain has been a leader, and his work has thrown much light not only on the special properties of the ionic micell, but also on the physical properties of colloidal particles as a whole. In the course of this work a great variety of experimental methods have been developed—chemical, electrical, optical and mechanical methods—which are novel in their application and are in some cases novel in themselves. Reference may be made to the quantitative study of "solubilization" and the elaboration of the air-borne ultracentrifuge in illustration of McBain's versatility of technique.

In addition to the composition and organization of a colloidal particle, its interface with the continuum has a decisive influence on its properties, and this gives special importance to the section of McBain's work which deals with the structure, composition and depth of macroscopic interfaces, especially of the surfaces of colloidal electrolyte solutions. Here also he has devised many methods of study, one of the most striking being the "microtome" method whereby the outermost layer of a solution can be peeled off with a rapidly moving knife blade so that the surface concentration of solute can be directly determined.

The HUGHES MEDAL has been awarded to Professor GEORGE PAGET THOMSON.

Thomson's researches have been spread over a wide range of experimental and theoretical physics.

Most of his earlier experiments were connected in some way or another with positive rays, and in this field he obtained a number of valuable results. Probably the most important of these is his discovery that the small angle scattering of protons in hydrogen could not be accounted for by treating the protons and electrons as point charges obeying the inverse square law of force, but that there must be some other law of force operating at the small distances of approach during the very close encounters involved.

The scope of his work in pure physics is indicated by the titles of three books he has written or, in one case, helped to write. These are: *The Atom*, *Wave Mechanics of the Free Electron* and the third edition of *Conduction of Electricity through Gases*. The last is a joint effort with his father and is the most important work there is on the subject. Within this range his work is both experimental and theoretical, but the experimental part predominates both in quantity and in importance.

Thomson has also made notable contributions to aeronautics. They include research work for the fighting air services during the Great War of 1914–18, a book entitled *Applied Aeronautics* 1919, and various contributions to Government publications.

Thomson's most distinguished work is based on Davisson's discovery—finally established in 1927—that electrons were reflected by single crystals as if they were possessed of the characteristics of waves. By brilliant experiments and able reasonings thereon, Thomson has opened out a new field of research which has been singularly fruitful and is still full of promise. He has been able to prove by direct experiment the correctness of Louis de Broglie's ideas of wave mechanics, not merely qualitatively but also quantitatively. In fact electrons of mass m and velocity v on passing through a crystal give a diffraction pattern identical with that given by a beam of X-rays of wave-length $\lambda = h/mv$, where h is Planck's constant, when passed in the same direction through the same crystal. In this way the lattice constants of the crystal can be measured instead of with X-rays, and in his first paper Thomson showed that the values he found using electron diffraction agreed to within one per cent with the values previously got by the use of X-rays.

CROONIAN LECTURE

Aspects of animal locomotion

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[Plates 1–7]

The power of locomotion is, perhaps, one of the most striking attributes of animal life. It occurs in all groups of animals and is characterized by two conspicuous features: (i) In no other biological activity is an animal brought into closer and more intimate contact with its environment. (ii) Closely related animals may display striking differences of locomotory pattern yet in every case the animal is able to deal precisely and efficiently with mechanical problems of great complexity.

For many years, the study of animal locomotion has been concerned with two, apparently distinct, types of problems. First, attention has been paid to the mechanical or kinematic principles which animals employ in order to progress from one place to another. In many terrestrial animals these principles are relatively simple, for their limbs represent levers of one type or another: in other cases the mechanical principles are more obscure—we know little concerning the kinematics of movement of a fish or a snail, and little or nothing of the forces which propel a bird actively through the air. These problems have long attracted attention and it is encouraging to know that they are now being attacked by methods as precise and as controlled as those employed by aeronautical or marine engineers. The second type of problem is of a different nature; it is concerned with the physiological nature of the locomotory machine. What is the nature of the neuro-muscular mechanism which enables an animal to utilize its muscular energy with such conspicuous precision and efficiency? How far are the movements dependent on the higher nervous centres, and how far are they dependent on the receipt of time signals from the outside world?

In practice these two types of problem are inseparable, for we cannot hope to analyse the physiological properties of a locomotory mechanism until we have a complete and accurate picture of all the forces acting on the body during each phase of its motion. It is, however, with the physiological rather than the kinematic aspect of locomotion that this lecture will be concerned.

A survey of the animal kingdom reveals that even the most divergent types of locomotory organs conform to two general principles: (i) Like all other voluntary movements, locomotion is effected by protagonistic and antagonistic muscles. (ii) Where more than one pair of muscles is involved, the order of their activity displays a regular and characteristic pattern. Recent studies of animal locomotion have been largely concerned with the nature of the mechanism which elicits from each pair of protagonistic and antagonistic muscles a rhythm of alternating activity; it has also been concerned with the mechanism which ensures that each of a series of muscles shall contract at an appropriate moment.

A clear understanding of the reciprocal relationship between the reflex activity of a protagonist muscle and that of its antagonistic partner was provided by Sherrington, who demonstrated that, in vertebrates, a stimulus which excites the motor neurone of one muscle produces, simultaneously, active inhibition of the neurones of its antagonist. This principle of reciprocal inhibition probably extends throughout the animal kingdom although its precise nature is not the same in all phyla. On the other hand, the nature of the mechanism which maintains the rhythm of activity between antagonistic and protagonist muscles remains obscure; nor do we know why each of a series of muscles undergoes contraction at an appropriate moment in the whole locomotory cycle. Two main theories have been advocated: (i) The muscular rhythm is regarded as the expression of a nervous rhythm which is generated in the central nervous system itself. (ii) The muscular rhythm is attributed to the receipt by the central nervous system of rhythmical stimuli arising in the peripheral sense organs of the active musculature. There is much to be said in favour of both these views, but before attempting to assess their relative merits it is convenient to consider how far certain regions of the central nervous system can be eliminated as essential parts of the mechanism.

THE ROLE OF HIGHER NERVOUS CENTRES

All modern work shows that, in the lower vertebrates, no essential locomotory role is performed by centres in the brain lying much anteriorly to the medulla oblongata. A fish, frog or lizard, whose brain has been transected anteriorly to the medulla, moves with full precision of movement (Steiner 1885); it can, of course, no longer adjust its path in response to any stimulus entering the nervous system anteriorly to the cut.

If, on the other hand, the nerve cord of a lower vertebrate is transected posteriorly to the medulla, very profound changes are at once observed in locomotory activity. The most obvious and most widely distributed of these changes is the failure of the animal to respond to stimuli which, under normal circumstances, would have elicited locomotion. Spinal transection induces, in all vertebrate types with one exception, a state of more or less profound spinal shock; the somatic musculature becomes limp and fails, partially or completely, to respond to exteroceptive stimulation. This depressed state may last for a few hours or days in a frog or a fish; it may last for several weeks in the higher mammals. Later, some recovery occurs and the animals begin to display characteristic patterns of reflex activity; never, so far as I know, is the full power of normal locomotion regained. The only exception to this rule is found in selachian fishes such as the dogfish (*Scyllium canicula*). This animal, after post-medullary transection, invariably displays, even when left undisturbed, an extremely persistent locomotory rhythm (Steiner 1885; Gray and Sand 1936) which can be slowed or accelerated by appropriate reflex stimulation (figure 1); comparable phenomena have occasionally been observed in the conger eel. In all other vertebrates the depressant action of post-medullary transection is very well defined and leaves no room for doubt that the medulla (or some slightly more anterior region of the brain) plays a role in the maintenance of full locomotory activity.

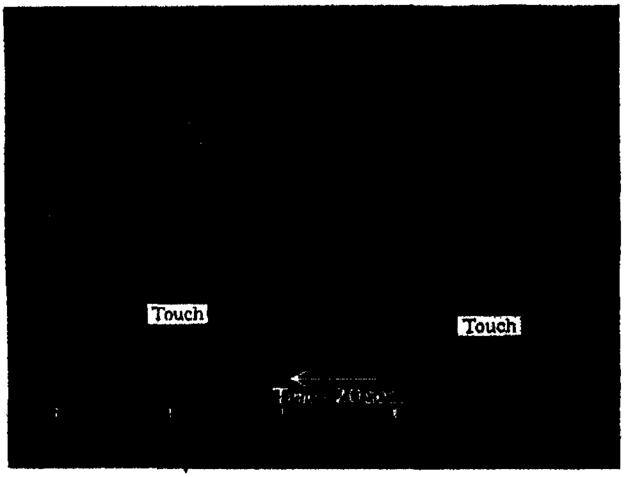

FIGURE 1. Persistent undulatory rhythm of the spinal dogfish (*Scyllium canicula*). Gentle tactile stimulation applied to the dorsal surface of the body increased both the frequency and amplitude of the movements. (From Gray and Sand 1936.)

Attempts to localize the locomotory areas in the hind brain of fish or amphibia involve considerable difficulties for two reasons: (i) There lie, within this region, nervous centres whose integrity is required for respiration; damage to the medulla involves, in nearly every case, severe respiratory disturbance. (ii) In many cases, the spinal centres associated with the brachial plexus appear to lie very near to the posterior end of the medulla, and it is difficult to keep them intact and at the same time avoid disturbance to the respiratory centres. According to ten Cate (1928), transection of the nerve cord between the second and fourth spinal nerves of *Triton cristatus* provides a spinal preparation free from medullary influences without at the same time abolishing reflex activity in the fore limbs. Working with similar material, Dr Lissmann and I have found it difficult to decide whether medullary or post-medullary transection of the nerve cord influences the activity of the fore limbs by damage to specific locomotory centres or by the general loss of tone which the transection induces in the anterior region of the cord.

The picture presented by mammalian material is similar, although it would seem that the critical levels in the brain lie somewhat anteriorly to those of fishes and amphibia. Laughton (1924) found that full co-ordination of all four limbs only occurred in the cat or dog so long as the posterior two-thirds of the thalamus lay behind the level of transection; in rabbits the critical regions appear to lie somewhat posteriorly to this level. Comparable results were obtained by Hinsey, Ranson and McNattin (1930). It will be noted that, in these cases, at least part of the fore brain is essential for normal locomotion; transections just posterior to the critical level cause decerebrate rigidity; sections posterior to the medulla yield spinal preparations in which locomotory activity is confined to the posterior limbs; as in the lower vertebrates, it is difficult to distinguish between the effect of spinal shock and destruction of specific centres. No systematic investigation has been made with reptiles: such an enquiry should yield interesting results, particularly as a test of the view that, during phylogeny, co-ordinating centres tend to move towards the more anterior regions of the vertebrate brain. For the time being, we must be content to remember that in all vertebrates (except selachian fishes) there lies in the medulla (or just in front of it) a region of the brain whose absence causes a profound depression in the level of locomotory activity; in tetrapods this depression is more marked in the fore limbs than in the hind limbs.

Although the posterior regions of the vertebrate brain are of considerable importance in the maintenance of full locomotory activity, they are not necessarily the loci of the centres which generate the rhythm of activity in locomotory muscles. This is clearly seen in the spinal dogfish and it may be recalled that, after sudden decapitation, some vertebrates exhibit—for short periods of time—a spontaneous state of active and highly co-ordinated locomotion (e.g. the eel, snakes and birds; see ten Cate 1936, 1937). Tarchanoff (1895) described normal swimming movements in a duck whose spinal cord had been severed between the third and fourth vertebrae. So far as I know there is no evidence of spontaneous tetrapodal locomotion after sudden spinal transection.

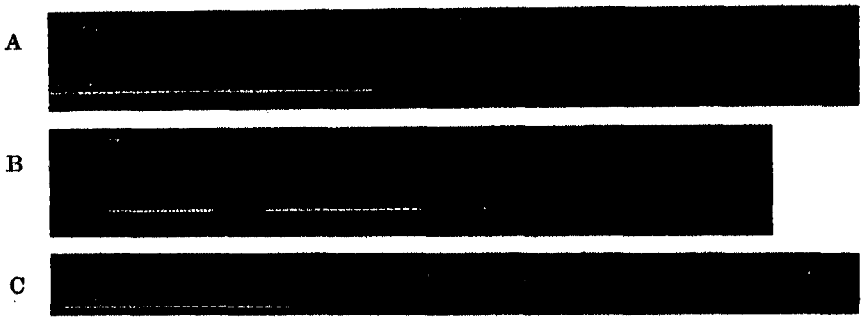

FIGURE 2. Locomotory rhythm induced in the decapitated body of a snake (*Tropidonotus natrix*) by application of repetitive electrical stimuli applied to the anterior end of the nerve cord. The frequency of the muscular rhythm varies with the intensity and frequency of the applied stimuli. In tracing A the frequency of the shocks was 4 per sec.; the resistance of the circuit was about 3000 Ω. In tracing B the voltage was constant at 34 V, the resistance was 3500 Ω and the current 9·5 mA; in the three successive periods of stimulation the frequency of stimulation was 5·5, 3·0 and 1·2 per sec. respectively. In tracing C the conditions were the same as in tracing B, but the frequency was constant at 4·0 per sec. and was applied during the periods marked above the tracing.

In a limited number of other cases, spontaneously inert spinal animals can be induced to display locomotory movements by the application to the nerve cord of a continuous series of appropriate artificial stimuli. This phenomenon was first observed by Sherrington (1931) during faradization of a small area in the lateral column of a decapitated cat. Comparable phenomena have been observed in the spinal eel (Gray 1936) and, recently, Dr Lissmann and I have elicited a well-defined locomotory rhythm in a decapitated snake by similar methods (figure 2). It must be admitted, however, that locomotion produced in this way is neither sustained for a length of time nor with a precision comparable to that displayed by normal animals; nor does it seem to occur in all vertebrates.

The elicitation of co-ordinated locomotory activity from low spinal vertebrates may be explained by the assumption that the whole of the essential mechanism for the maintenance of a locomotory rhythm is located behind the medulla: when an eel or snake is suddenly decapitated the transitory violence of peripheral stimulation is, presumably, sufficient to overcome the depressant effect of the earlier stages of spinal shock. If this interpretation be correct, one may look upon the medulla as a "Stimulations organ" (see Wolsky 1933) which maintains the excitability of the spinal cord at a level which enables the latter to display its full locomotory activity when exposed to extraneous stimuli of a comparatively low level of intensity. In the dogfish we must, for the time being, assume that the medulla exerts, whilst the intact animal is at rest, an inhibitory rather than an excitatory effect. It is obvious, however, that the influence of the medulla and of associated regions of the brain on the locomotory activities of vertebrates is still very far from clear and well deserves further study.

Invertebrate animals provide a large number of instances in which removal of the higher nervous centres fails to abolish, or seriously disturb, co-ordinated locomotory movements. Earthworms, some insects (Bethe 1897; ten Cate 1928; Fraenkel 1932) and crabs all move normally after removal of the "cerebral" ganglia; after complete decapitation a leech swims persistently (von Uexküll 1905; Gray, Lissmann and Pumphrey 1938), as does a cuttlefish (Sepia) after severance of the connexions between the sub-oesophageal and supra-oesophageal ganglia (Dr J. Z. Young—personal communication).

A careful analysis of the relevant facts and of the diversity of results obtained from different species of animals would be of extreme interest, but, so far as they go at present, the data suggest that a normal pattern of locomotion can, to a surprising extent, be displayed in the absence of the higher centres of the nervous system. We may now consider how far it is dependent upon the integrity of peripheral sense organs.

THE ROLE OF PERIPHERAL EXCITATION

During normal locomotion, the limbs and sometimes the body of a terrestrial animal are in periodic contact with the ground and each of these contacts sets up a stream of afferent impulses which travel into the nerve cord. Similarly, each movement of a locomotory muscle is liable to excite proprioceptor sense organs within these muscles or in neighbouring muscles, joints, or tendons. For reasons which will be considered later, Graham Brown (1912) and Sherrington (1913) concluded that the presence of peripheral sense organs was not an essential requirement for locomotory activity in mammals. On the other hand, a wider survey of the whole animal kingdom makes it difficult to accept this conclusion in an unqualified form.

If peripheral activity is unnecessary for the expression of a locomotory rhythm, the latter should express itself under circumstances in which the nerve cord is, so far as possible, isolated from all patterns of peripheral stimulation comparable to those which it receives during normal ambulation. Observations of this type indicate that the great majority of animals fail to exhibit typical ambulatory activity unless supplied with the normal background of peripheral stimulation. Examples are provided by the leech (*Hirudo medicinalis*), the myriapod (*Doratogonus annulipes*), many insects, newts and the snake (*Tropidonotus natrix*).

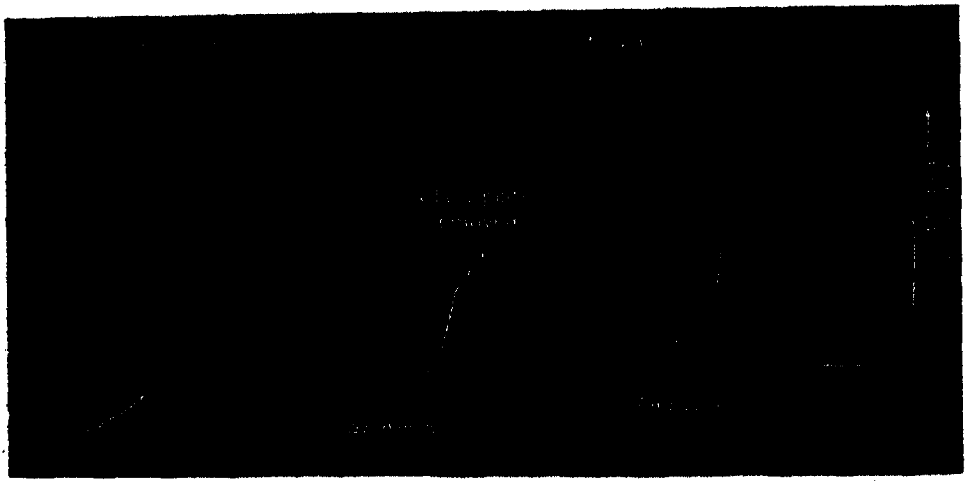

FIGURE 3. Record of a leech, after denervation of both suckers, walking on a horizontal glass plate. Note cessation of ambulation as soon as contact with the plate is removed; when freely suspended, the preparation showed typical swimming movement until ventral contact with the plate was again established. The rapid swimming rhythm can just be detected from the waviness of the tracing. The record reads from right to left. (From Gray, Lissmann and Pumphrey 1938.)

Terrestrial locomotion in the leech is carried out by alternate contractions of the longitudinal and circular muscles, and the activity of each set of muscles is ushered in by the attachment to the ground of the appropriate sucker; Gray *et al.* (1938) have shown that the ambulatory rhythm is dependent on the maintenance of effective contact between the suckers, or of the ventral surface of the body, with the ground. If this contact be removed, ambulation ceases and a totally different pattern of muscular activity is set up—namely the pattern characteristic of the free swimming animal (figure 3). If, on the other hand, we supply the ventral surface of the body (or the sucker) of the free swimming animal with gentle tactile stimulation from a solid surface, the muscular pattern requisite for ambulation is at once resumed. Comparable phenomena are very common among other invertebrate animals, and it is interesting to note the case—observed by Dr Lissmann—of the nemertine (*Cerebratulus marginatus*) in which one region of the body will exhibit typical ambulatory peristalsis whereas the remainder, not in contact with the ground, exhibits the undulatory pattern characteristic of swimming (figure 4, plate 1). Terrestrial invertebrates provide analogous facts; so long as the limbs of the myriapod (*Doratogonus*) are in contact with the ground they exhibit, during activity, a peculiarly well-defined and co-ordinated rhythm in which each limb occupies a definite posture relative to its neighbours: when contact between the limbs and the ground is removed, all co-ordination between individual limbs is lost (see p. 42). So again, in insects (Fraenkel 1932)—a fly (e.g. *Muscina stabulans*), freely suspended in air, flies persistently, but if the tips of the legs are brought into contact with a solid object the motion of the wings ceases until this contact be removed.

Among vertebrates, the newt or frog provides an example of the same principle. A newt, freely suspended in water, responds to gentle stimulation by the undulatory movements characteristic of normal swimming; these movements cease as soon as one or more limbs encounter a solid object; when all four limbs are on a solid object, a stimulus, which applied to the freely suspended animal elicits swimming, now elicits typical ambulation. In carnivorous mammals, tactile stimuli are sometimes without locomotory significance: Sherrington (1910) showed that desensitation of the paws did not reduce the precision of movement of a cat (see p. 36), whereas in the monkey (*Macacus rhesus*) desensitation of the skin of the paw apparently causes considerable dislocation of movement (Mott and Sherrington 1895). In mammalian locomotion, however, proprioceptor stimulation is probably dominant.

These examples show that locomotory patterns of muscular activity can, with few exceptions, only be displayed against a pattern of peripheral stimulation which is identical with, or at any rate very similar to, that which occurs under natural conditions. That locomotory movements should cease during periods of unsuitable environmental conditions is obviously of functional significance.

THE CENTRAL THEORY OF THE LOCOMOTORY RHYTHM

It is, at this point, convenient to consider more precisely the influence of peripheral excitation on the locomotory centres in the central nervous system. In the first place, it is conceivable that this excitation plays its part in raising the level of the excitability of the nerve cord to that necessary for the expression of the cord's intrinsic powers of rhythmical activity. This view has been mentioned recently by Adrian (1939) in his Ferrier Lecture and seems to imply that the particular pattern of peripheral excitation is, perhaps, of less importance than its general level of intensity. On the other hand, it is conceivable that the efferent rhythm emerging from the nerve cord is itself determined by an extrinsic rhythm arising in the peripheral nerve endings in the skin or in the locomotory muscles themselves.

Among mammalian physiologists, these alternatives have been obvious for at least twenty-five years, and a discussion of their respective merits is associated with the names of Philippson (1905), Graham Brown (1912, 1913) and Sherrington (1913, 1931). Philippson was the advocate of a peripherally determined rhythm against which Graham Brown and Sherrington marshalled very strong evidence. Sherrington showed that a cat, whose paws and ankles had been entirely desensitized, could walk over the rungs of a ladder with full precision of movement. If a peripheral rhythm is fundamental for mammalian locomotion, it must arise in the proprioceptor sense organs of the muscles, tendon and joints, and not in the tactile receptors lying in the skin. In 1912 Graham Brown completely deafferentated a pair of antagonistic muscles (tibialis anticus and gastrocnemius) in the cat, and showed that suitable electrical stimuli, applied simultaneously to the saphenous nerves, induced rhythmical and alternating contractions in the two muscles. In the following year, Sherrington (1913) showed that reciprocal stepping could be induced by similar methods in the isolated and deafferentated right and left vastocrurii (extensor) muscles of a decerebrate cat. Graham Brown (1913) also observed spontaneous stepping in the limbs of a cat under a depth of anaesthesia sufficient to extinguish response to reflex stimulation. These observations constitute the foundations of the central theory of locomotor rhythms to which support was provided by the discovery of rhythmical outbursts of electrical activity in the totally excised nervous systems of both vertebrates (*Carassius*, Adrian and Buytendijk 1931) and invertebrates (*Dytiscus*, Adrian 1931). Quite recently von Holst (1937) has described similar rhythms in the excised nervous system of the earthworm.

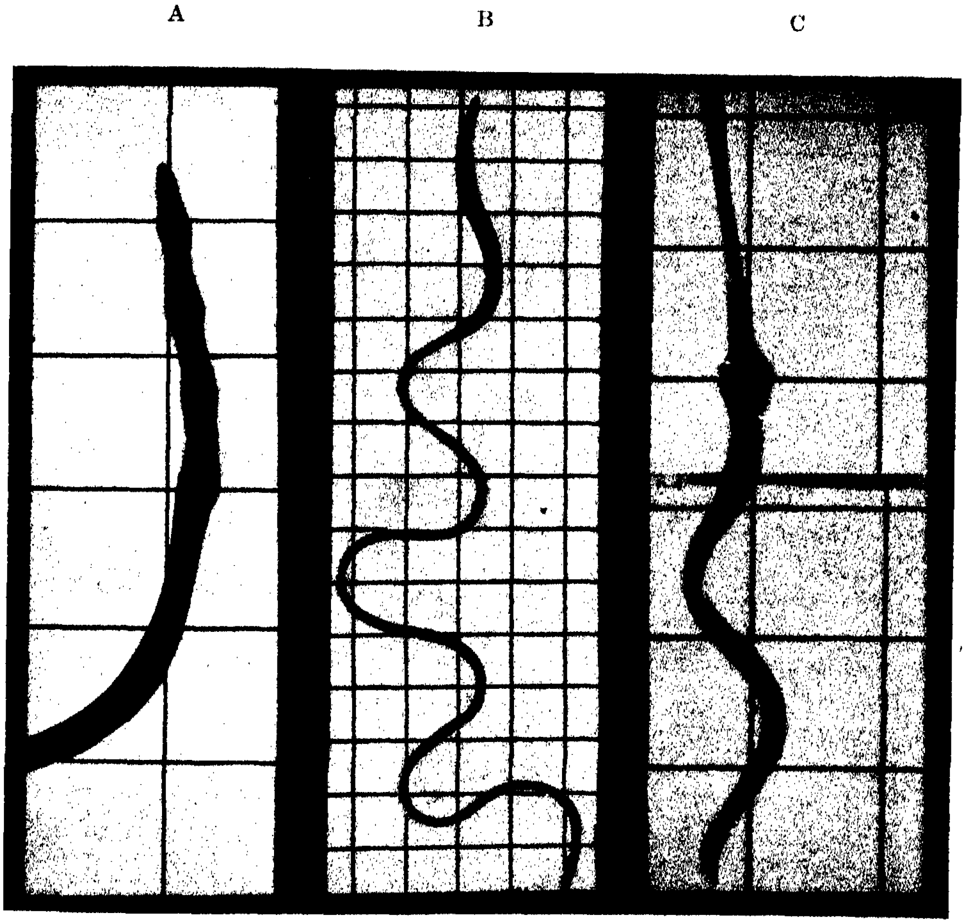

FIGURE 4. *Cerebratulus marginatus.* A. Creeping over a solid surface—note peristaltic waves. B. Swimming freely in water—note undulatory movement. C. A specimen suspended vertically with the posterior end only in water—note peristalsis in anterior region and undulatory movement in posterior end. (From photographs by H. W. Lissmann.)

Adrian associated the rhythms observed in the goldfish with respiratory activity, so that von Holst's observation is, perhaps, more pertinent as he associates the rhythm in the isolated nerve cord of the earthworm with the locomotory movements seen in an intact worm. The advocates of the central theory clearly recognize that a rhythm arising in centres within the nerve cord must be influenced by particular patterns of peripheral stimulation, but they maintain that these influences are not essential for the production of the rhythm itself—in the words of Graham Brown they "play a regulating and not an intrinsic part in the act" of locomotion.

Although the experimental evidence is imposing, the concept of a centrally determined rhythm is not entirely free from difficulty. In the first place, conclusions drawn from observations of totally excised nervous systems must be received with caution, for the degree of injury involved may yield a highly distorted picture of normal activity. In order to retain a reliable index of locomotory activity in an isolated nervous system it is desirable to use a preparation in which it is possible to test the state of muscular activity just before the isolation of the whole nervous system is complete. A preparation of this type is available in the leech (*Hirudo medicinalis*) (Gray *et al.* 1938). So long as a few segments of the musculature of this animal retain their nerve supply and are performing active swimming movements, the whole nerve cord is maintained in a state of perfectly co-ordinated and rhythmic electrical activity (figure 5). As soon as the nerve cord is completely isolated from the intact and active musculature, all rhythmic activity ceases in the isolated cord. Attempts to revive a state of electrical rhythm by artificial stimuli failed, but it is obviously possible that the nature of the stimuli may have been inadequate. In isolated cords of the earthworm Dr Lissmann and I (1938) have confirmed the presence of electrical rhythms, but we did not feel satisfied that, as observed by us, their frequency and duration were such as made it reasonable to associate them with rhythms of peristalsis in normal animals.

An attempt to test the central nature of the normal locomotory rhythm has been made by other methods. If the fundamental rhythm of flexor and extensor activity in the limb of a terrestrial vertebrate is determined by centres in the nerve cord which are only dependent, to a secondary degree, upon events occurring in the peripheral nervous system of the limb itself, then complete deafferentation of limbs should not necessarily destroy the power of co-ordinated rhythmical movement. Observations on the behaviour of deafferentated limbs have been made by numerous authors and there seems general agreement that a mammalian limb when deprived of its sensory nerves fails to take its normal part in locomotion.

Mott and Sherrington (1895) found that total deafferentation of single fore or hind limbs of a monkey (*Macacus rhesus*) caused obvious lack of co-ordination and, except under conditions of stress, the limb took no part in progression or prehension. On the other hand, when the motor cortex was explored electrically, well-defined and co-ordinated movements occurred as clearly in the deafferentated arm and hand as in the intact limb. Equally obvious locomotory disturbances are observable in single deafferentated limbs of cats and dogs (Bickel (1897), Ranson (1928)). In no case does a clearly defined locomotory rhythm appear in a deafferentated

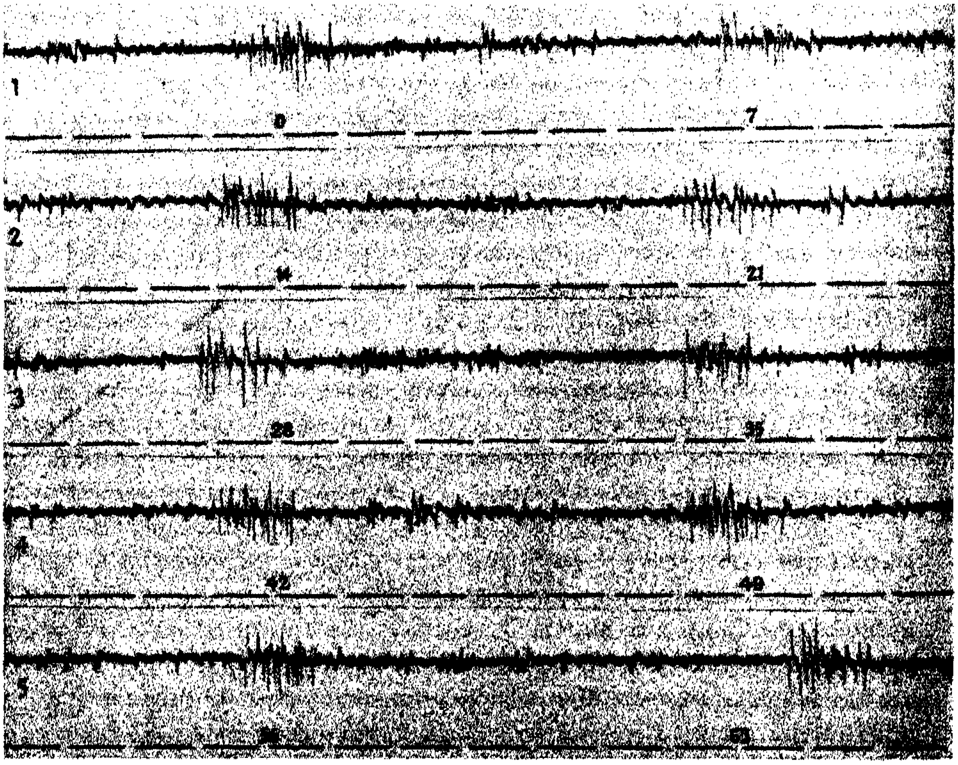

Figure 5. Continuous record of electrical activity in the proximal region of a severed segmental nerve of a decapitated leech (*Hirudo medicinalis*). All nerves in the neighbourhood of the recording nerve were cut, only nerves in the posterior region of the body being intact. So long as the muscles in the latter region exhibited active swimming movements, the severed nerves in the anterior end of the body exhibited an electrical rhythm identical in frequency with that of the muscular rhythm seen in the intact posterior segments. Time is shown in $\frac{1}{10}$ sec. (From Gray *et al.* 1938.)

limb. So far as mammals are concerned, we seem therefore to reach a paradoxical position. Under experimental conditions it is possible to elicit a locomotory rhythm in a pair of completely deafferentated muscles; the animal, on the other hand, is unable to produce a comparable phenomenon.

Deafferentation of the limbs of other vertebrates yield results in striking contrast to those observed in mammals. Trendelenburg (1906) found that unilateral deafferentation of the fore limbs of a pigeon did not seriously disturb the power of flight; so long as the wings were engaged in active movement, the deafferentated limb behaved quite normally. After bilateral deafferentation the bird could no longer fly. So far as I know, no evidence is available from reptiles, but all observers are agreed that deafferentation of single limbs in amphibia causes no obvious disturbance in the locomotory functions of that limb or of its fellows. It has also been known for many years that bilateral deafferentation of the hind limbs of a frog does not abolish the power to swim in water. Weiss (1936) found that toads (*Bufo americana* and *B. fowleri*) could maintain fully co-ordinated ambulation after complete deafferentation of both hind or of both fore limbs; a result which was unexpected in view of Detwiler and Vandyke's (1934) observation that a limb of the axolotl which has developed without any sensory nerve supply fails, subsequently, to take any part in locomotion. Concerning the effect of more extensive deafferentation of tetrapod vertebrates very little evidence has hitherto been available. Weiss deafferentated all four limbs of two toads and found that it was very difficult to induce the animals to move. Occasionally the animals moved for a few steps with the hind limbs alternating as in walking; jumping was rare and, if it occurred, it was not effective. Nevertheless, Weiss concluded that his experiments "emphasized the central autonomy of the motor (locomotory *sic*) patterns and their wide independence of proprioceptor control in amphibia". A similar conclusion was reached by von Holst (1935) in the case of fish; von Holst severed all the dorsal roots posterior to the pectoral fins of the tench (*Tinca vulgaris*) and found that the animal was still capable of executing swimming movements when stimulated through the pectoral fins.

The behaviour of deafferentated musculature thus appears to differ in different groups of vertebrates and it is possible that during phylogeny a primitive type of central control of ambulatory activity has been dominated by a secondary peripheral type, as is suggested by Weiss. We still have, however, difficulty in explaining the rhythms in deafferentated mammalian muscles as observed by Graham Brown and Sherrington. Before accepting this position as final it seemed desirable to explore the effect of extensive deafferentation in lower vertebrates in greater detail. For experiments of this type the toad (*Bufo bufo*) is particularly suitable; the normal pattern of ambulation is extremely constant (see p. 48 and figure 11) and the animals survive the effect of operations with considerable fortitude. Dr Lissmann and I have investigated the effect of deafferentation of these animals in considerable detail* and I would like to take this opportunity of paying a tribute to Dr Lissmann's operative skill, without which these observations would not have been possible.

The results of these experiments can be summarized as follows (see figure 6, plate 2):

(i) After deafferentation of any single limb the animal, when walking, continued to use this limb normally; the deafferentated limb showed a perfectly normal rhythm of flexor and extensor activity and took its proper place in serial relationship to the other limbs. When the animal was at rest, the operated limb could be placed passively by the operator in any position and remained there; as soon as the animal moved, whether in response to visual, labyrinthine, or mechanical stimulation, the limb behaved with apparently complete precision of movement. These observations confirm those of other workers and there can be little doubt that they present a striking contrast to those obtained with mammalian material.

(ii) After deafferentation of two limbs the picture presented differed somewhat according to the limbs selected for experiment. After deafferentation of both hind limbs, the animals walked with a perfectly normal rhythm of movement and the intensity of stimulus required to initiate movement was not markedly greater than that in intact animals. When at rest the two hind limbs were held in the usual flexed position but offered no resistance or response to passive displacement. Similar behaviour was characteristic of animals in which one hind and one fore limb had been deafferentated.

After deafferentation of both fore limbs the normal rhythm of walking was retained, but the operated limbs showed definite loss of muscular tone both during movement and when at rest. Whilst at rest, the fore arm rested on the ground and usually remained so during the extensor phase of the step: the limb thus failed to act, to its normal extent, as an effective prop on which the body was swung forward by extension of the contralateral hind limb. So far as could be seen, the disturbance in the course of locomotion of such animals was due to the mechanical effects caused by the

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* We were unaware of Weiss's (1936) work until after the completion of our experiments.

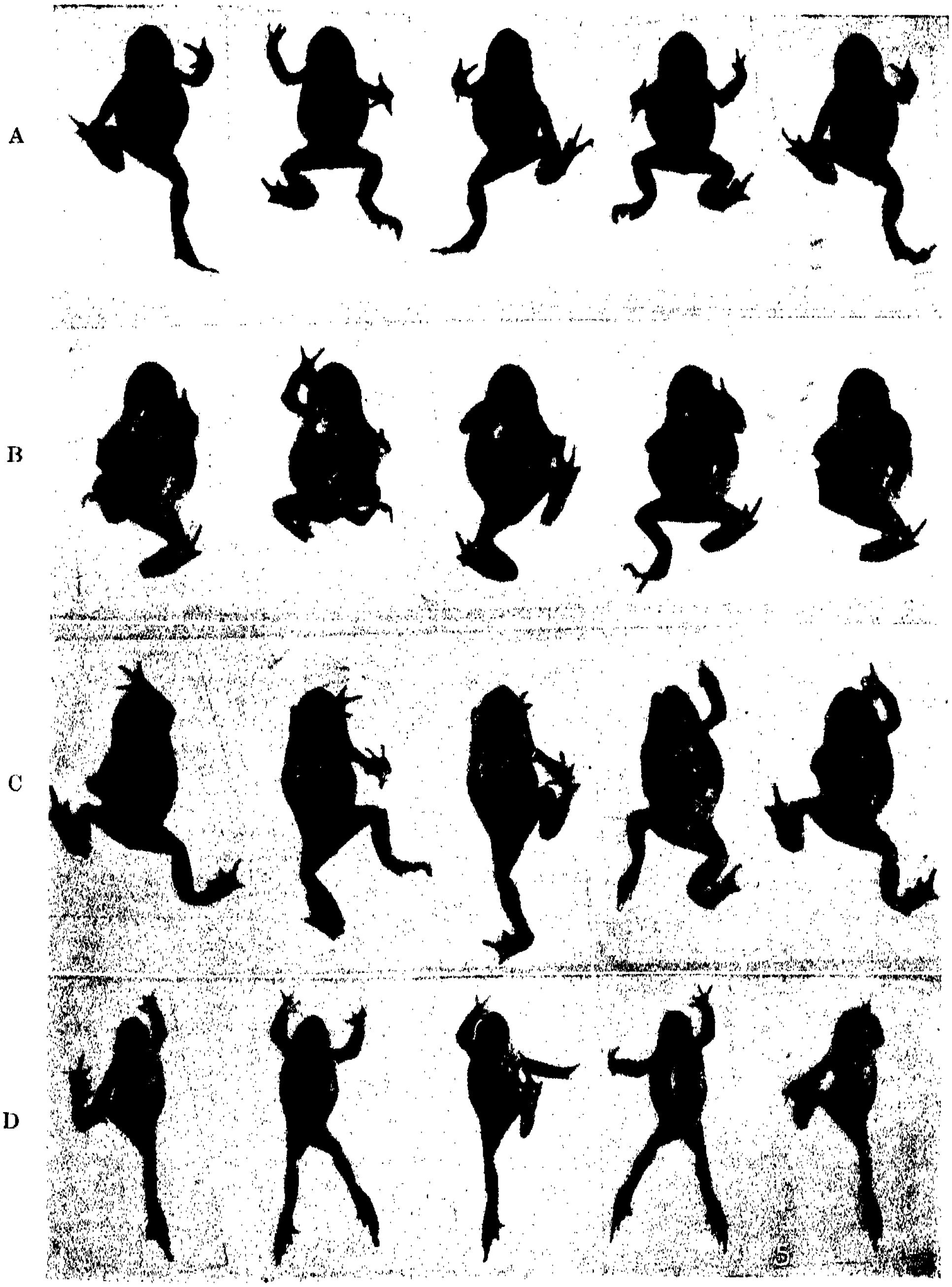


Figure 6. Corresponding phases in the step of: A. Intact toad. B. Toad after deafferentation of both hind limbs. C. Toad after deafferentation of both fore limbs. D. Toad after deafferentation of all four limbs. Note the ungainly attitude of the limbs after deafferentation of all four limbs.

(*Facing p.* 40)

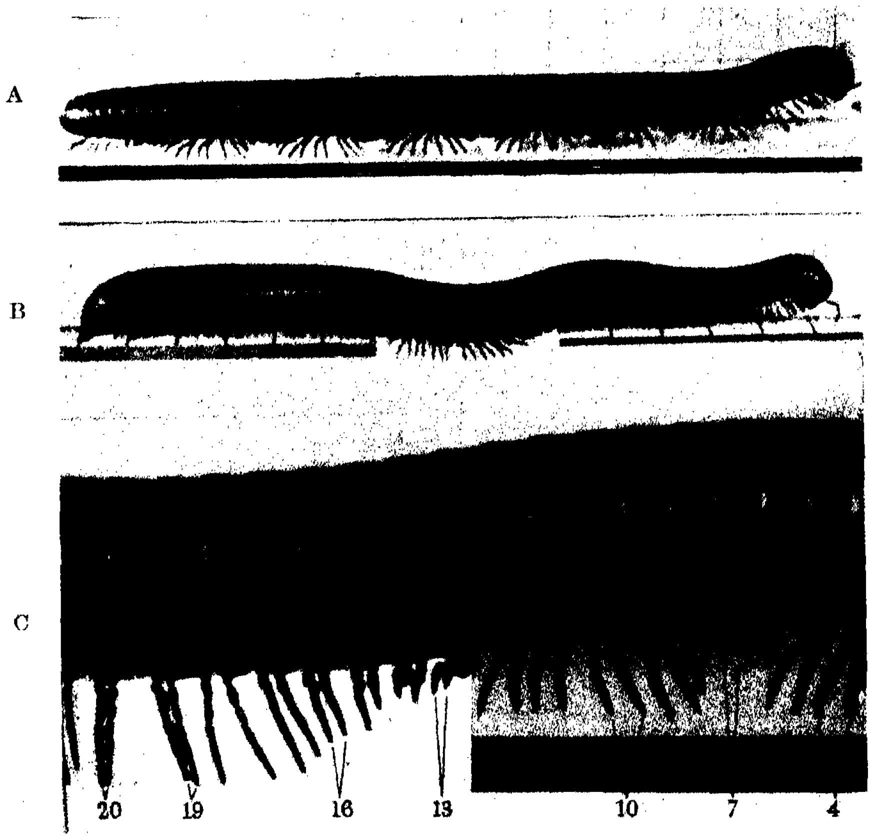


FIGURE 7. A. Intact specimen of the myriapod (*Doratogonus*) walking to the right on a horizontal surface. Note the regular wave pattern displayed by the limbs. B. Similar specimen moving across a gap 4 cm. wide. Note the loss of wave form in the centre of the gap. The legs in contact with the substratum retain their normal undulatory co-ordination but this is not well shown in the photograph. C. Enlargement of a region of the body in the vicinity of the leading edge of a 4 cm. gap. Note that the limbs lying remotely from the substratum (e.g. segments 20, 19) show no co-ordinated movements whilst those lying near the edge of the substratum (segments 16–13) take up their correct relationship to limbs in contact with the substratum (segments 12–4); the latter limbs exhibit the typical wave pattern. The segments are consecutively numbered from an arbitrarily chosen segment in the anterior part of the animal.

loss of tone in the muscles of the fore arm and wrist. In many cases the fore limbs were thrust forward to an abnormal extent during the flexor phase of movement.

(iii) After deafferentation of three limbs quite definite loss of tone was observable in the operated limbs, yet in all cases the animal was able to progress. In some cases the normal rhythm of the limbs was retained—in others some disturbance was observable, partly owing to the long latent period in the response of individual limbs and partly to the mechanical effects caused by (*a*) the failure of the fore limbs to act as efficient props, (*b*) the tendency of the hind limbs to slip—the latter phenomenon being due, apparently, to lack of tone in the muscles which normally press the planta on to the surface of the ground.

(iv) After deafferentation of all four limbs, the animals did not readily respond to external stimulation—but if left in a tank they invariably walked after latent periods varying from ½ to 5 min. towards a shaded area. In most cases progression was laboured and ungainly, the limbs being thrust out to an abnormal extent and often slipping on the surface. Under favourable conditions, the normal rhythm of limb movement was retained but its smoothness and regularity had gone, its frequency was very slow, the body was dragged along the ground, the animals quickly tired and the loss of muscular tone was very obvious.

(v) After bilateral deafferentation of all the post cranial nerves no animal exhibited co-ordinated walking. More or less ineffective righting reactions were observed and a limited number of well co-ordinated swimming movements were made in water. If left undisturbed, the animals were lethargic, but limb movements were frequently observed when the head of the animals had been facing a shaded area for some period of time.

It seems clear from these facts that whereas a limited amount of deafferentation can occur without seriously impeding the activity of any of the locomotory musculature, there comes a time when further deafferentation causes very marked and permanent disturbance in the locomotory rhythm; locomotory activity of an animal deprived of all peripheral stimulation from its spinal nerves is, if not completely absent, unco-ordinated to a very marked extent.

The interpretation of these facts is not easy. We may either conclude that the whole of the locomotory musculature can act as a co-ordinated whole as long as a limited fraction of it retains the sense organs necessary for initiating a peripheral locomotory rhythm; alternatively, it is possible that as deafferentation becomes more and more extensive so the tone of the central nervous system is progressively lowered until it can no longer display its intrinsic rhythmical properties. Dr Lissmann and I have attempted to assess the relative merits of these two alternatives by deafferentation of all four limbs of a toad and section of the ventral roots of the remaining spinal nerves. Four preparations of this type have been made and all failed to show any sign of co-ordinated ambulation either with or without extraneous stimulation of the body wall; in at least one case quite active and co-ordinated swimming occurred when the animal was placed in water.

We, therefore, incline to the view that a co-ordinated ambulatory rhythm is dependent upon the integrity of the sensory nerve supply from part at least of the somatic musculature; if further work should confirm the fact that sensory stimulation from the muscles cannot be replaced by a rhythmical stimulation from the skin, it will be legitimate to infer that the contraction of these muscles sets up proprioceptor impulses which play the role of time signals in the maintenance of the rhythm in the whole of the locomotory musculature. We must admit that we have no definite evidence which indicates a participation of the trunk musculature in the locomotion of a normal adult toad, but it is interesting to remember that these muscles undoubtedly take part in the movements of the larva and of all primitive adult vertebrates.

In view of the very limited extent of intact musculature required for the maintenance of a definite degree of co-ordination in the limb movements of *Bufo*, it is important to note that in certain of Graham Brown's experiments no intact afferent nerves were present. On the other hand, it is conceivable that the movements observed by von Holst in the deafferentated tench were due to the fact that a limited number of dorsal roots were intact in the neighbourhood of the pectoral fins. Only further work can decide how far these suggestions have any real weight; in the meantime it is significant that Dr Lissmann has, quite recently, shown that complete deafferentation of the muscles of a spinal dogfish abolishes the persistent rhythm so characteristic of this preparation; here again, however, it is difficult to distinguish clearly between deprivation of proprioceptor activity and general loss of central nervous excitability.

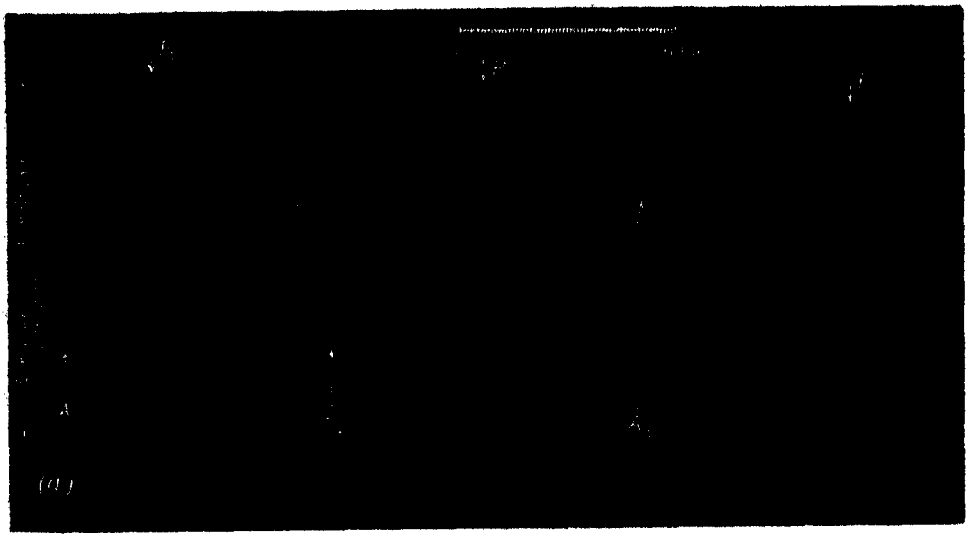
Phenomena comparable to those displayed by a partially deafferentated toad or leech are to be seen in the myriapod (*Doratogonus*). When all the limbs of the moving animal retain normal contact with the ground, their postural relationship to each other is such as to give the impression of waves of activity passing from the posterior to the anterior end of the animal (figure 7, A, plate 3); this co-ordination is lost if none of the legs are allowed to touch the ground. By compelling the animal to cross an appropriate gap, between two supports, some of the limbs are liberated from contact with the ground; by varying the width of the gap it is possible to see that so long as a limb, not in contact with the ground, is separated—by a distance not greater than about four segments of the body—from one which is in normal contact with the ground, the former limb maintain sits correct posture relative to its normal neighbours; if the distance is greater than four segments co-ordination is lost (figure 7, B, C).

The simplest, but not necessarily the correct, interpretation of the available facts suggests that a relatively small region of musculature (in *Bufo*, *Hirudo* and *Doratogonus*) which is sending its normal pattern of peripheral excitation into the nerve cord can impress upon more distant motor centres in the cord patterns of activity which enable their associated muscles, although themselves deafferentated, to display fully co-ordinated locomotor activity.

For the present it is necessary to suspend judgment concerning the existence of locomotory activity in the absence of all tactile and proprioceptor time signals, but it is clear that, when such stimulation is entirely withdrawn, the locomotory powers of an animal are reduced to a very low level. We may now approach the problem from a somewhat different angle.

THE ROLE OF PERIPHERAL REFLEXES IN LOCOMOTION

If the nature and frequency of the locomotory movements is determined by the nature and frequency of stimuli coming from peripheral sense organs, it ought to be possible to control the locomotory movements by appropriate modification of the peripheral stimuli. Suitable material for such experiments is provided by the leech, in which progression is effected by alternate contractions of the longitudinal and circular muscles. The contraction of the circular muscles is ushered in by attachment of the posterior sucker to the substratum, and the contraction of the longitudinal muscles by attachment of the anterior sucker. When a leech is suspended in air and maintained under light tension, it is possible to maintain and control a wide variation of frequency in ambulatory movements by providing an appropriate variation in the time elapsing before a sucker is allowed to attach itself to a small piece of coverslip (Gray *et al.* 1938) (see figure 8). The protrusion of the anterior sucker depends on the isotonic contraction of the circular muscles and the protrusion of the posterior sucker on the isotonic contraction of the longitudinal muscles. Further, the duration of the contraction phase of the longitudinal muscles can be controlled by varying the load under which they contract (see



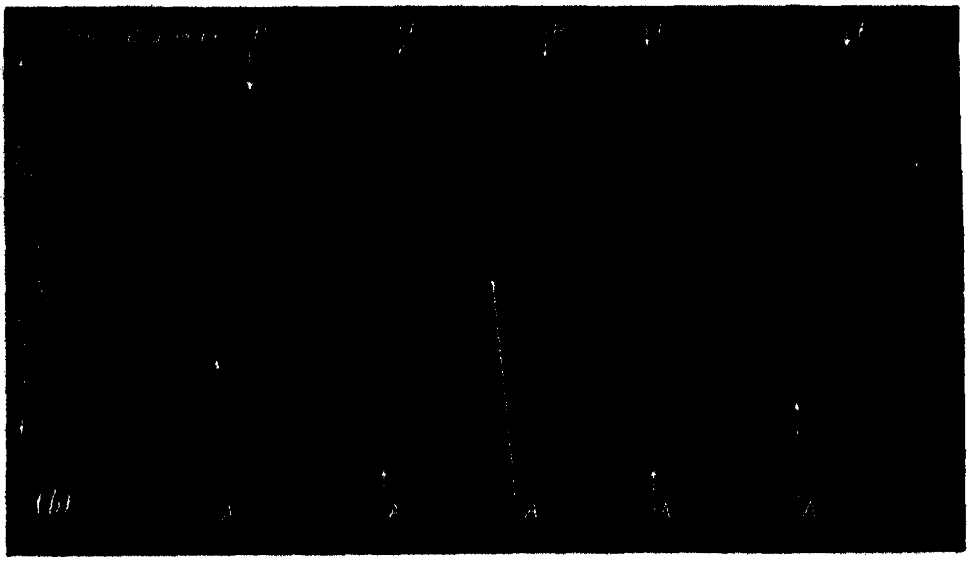


FIGURE 8 *a*, *b*. Records (reading from left to right) of an intact leech suspended in air to an isotonic lever. At *A* the anterior sucker was allowed to attach itself to a small piece of coverslip, shortly after which the posterior sucker—if attached—detached itself spontaneously. At *P* the posterior sucker was allowed to attach itself to a small piece of coverslip, whereupon the anterior sucker—if attached—detached itself spontaneously.

Note: (i) The contraction of the longitudinal muscles which follows attachment of the anterior sucker. If attachment of the anterior sucker is effected when circular contraction is restricted to the more anterior segments of the body, the first effect of fixation is complete longitudinal relaxation throughout the body, and this is then followed by a complete wave of longitudinal contraction throughout the whole body. (ii) The rapid and complete relaxation of the longitudinal muscles on attachment of the posterior sucker. In all cases relaxation of the longitudinal muscles is accompanied by contraction of the circular muscles and vice versa. (From Gray, Lissmann and Pumphrey 1938.)

figure 9). The whole cycle of ambulation in the leech appears to present a clear picture of a rhythm timed by signals from the periphery. It must be remembered, however, that a leech will still walk after denervation of both suckers if its ventral body wall is allowed to be in contact with the ground, so that more than one type of peripheral signal must be available. Similarly, a blind man walking over an irregular surface replaces, by tactile signals, the visual control employed by a normal individual.

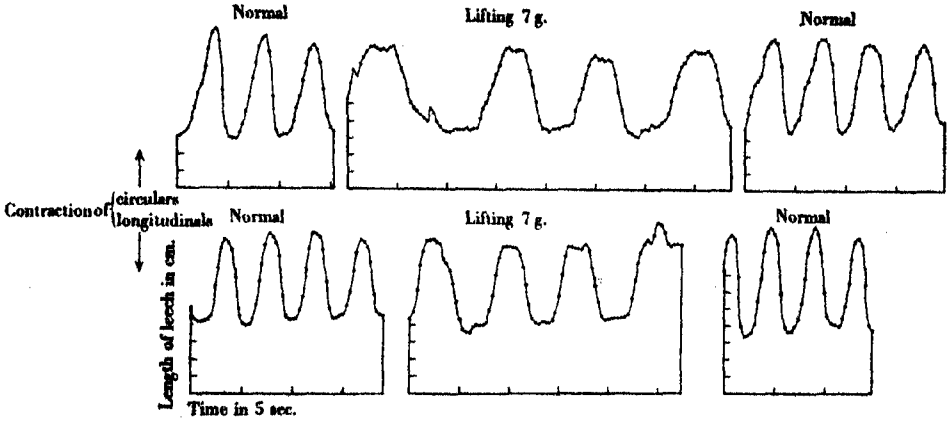


FIGURE 9. Graphs obtained from cinematograph records, showing the effect of loading on the frequency of the ambulatory rhythm in the intact leech. Note that the frequency is decreased by increasing the amount of work performed by the longitudinal muscles. The lower record was obtained from a different animal to that used in the upper record. (From Gray, Lissmann and Pumphrey 1938.)

Another instance of the effect of peripheral events on locomotory frequency and pattern is provided by a myriapod. An animal (*Doratogonus*) walking along a level surface at a velocity of 54 cm./min. showed a co-ordination of limb movements such that each "wave-length" on the body was 1·86 cm. (9 segments) and the frequency of limb movement was 38 per min. The same animal walking up an incline of 32° to the horizontal moved at a velocity of 19·2 cm./min. and at the same time the frequency of limb movement fell to 19 per min. and the wave-length rose to 2·6 cm. (12 segments). These facts show clearly that the frequency and form of the locomotory pattern can be readily controlled by environmental conditions.

Other evidence, supporting the view that peripheral events may be responsible for the phasing of locomotory movements, is available in a number of animals in which it can be shown that the isotonic contraction of one set of muscles automatically provides a mechanical stimulus capable of exciting the next muscle in the series. Phenomena of this type are found in the earthworm (Friedländer 1888; Gray and Lissmann 1938). The same general impression follows from the fact that the locomotory response of a vertebrate limb to extraneous stimulation depends on the *initial state* of passive posture of the limb.

INFLUENCE OF LOAD ON THE CO-ORDINATION OF LIMB MOVEMENT IN THE MYRIAPOD (*DORATOGONUS*)

Angle of inclination of path	Gravitational load g.	Wave-length in cm.	Frequency of limb movements per min.	Velocity of waves relative to body cm./sec.	Velocity of animal relative to ground cm./sec.
0°	0	1·86	38	1·17	54
22°	3·7	2·6	25	1·06	27
32°	5·3	2·6	19	0·84	19·2

Magnus (1909) showed that a stimulus applied to one limb of a spinal dog would induce extension in a passively flexed contralateral limb, but would induce flexion if the contralateral limb were originally in a state of passive extension. Both Magnus (1909) and Sherrington (1910) observed that the opening phase of stepping in the limb of a suspended spinal dog depends upon its initial state of passive posture. Precisely similar phenomena can be seen in a spinal newt. If such a preparation be tapped on the tail when one limb is in a state of passive flexion, that limb extends; if the tail is tapped when the limb is in a state of almost complete passive extension, the limb responds by flexion. Similar phenomena can be observed in intact newts and many other instances from invertebrate material have been recorded by von Uexküll (1929). Proprioceptor activity is almost certainly involved in all these cases and it is difficult to resist the conclusion that the opening phase, at least, of the normal locomotory rhythm is determined by peripheral events. In this connexion it is interesting to note that passive displacements of locomotory appendages have been shown to excite activity in their sensory nerves, e.g. in the pectoral fins of the skate (Fessard and Sand 1937).

It may be noted that so long as an animal is progressing over a level surface, the concept of a centrally controlled locomotory rhythm presents no practical difficulties; the theory is less satisfactory when applied to movement over an uneven substratum or when the resistance to movement varies. That the locomotory rhythm should be largely affected by environmental conditions is clearly necessary for efficient progression, and the facts suggest that peripheral stimuli, impressed on the animal by its environment, if not of fundamental importance as pacemakers of the ambulatory rhythm, are, at any rate, capable of modifying any underlying central rhythm to an extent which must render the latter almost unrecognizable.

THE ANALYSIS OF THE LOCOMOTORY CYCLE IN TERMS OF PERIPHERAL REFLEXES

The concept of the ambulatory cycle as a closed chain of peripheral reflexes is based on the idea that when a limb responds to stimulation by active movements it generates, in its own sensory nerves, impulses which not only affect its own behaviour but also that of neighbouring limbs. In the limbs of all tetrapod vertebrates the flexion reflex is the dominant characteristic of the spinal animal. A wide variety of stimuli applied to a limb, or artificial stimuli applied to the central end of its sensory nerves, induces flexion of the limb. Our present problem is to decide: (i) how far this reflex can be elicited by stimuli, comparable in form and intensity, to those automatically set up during normal locomotion, and (ii) how far the flexion reflex induces, in other limbs, responses which are clearly of locomotory significance. For the foundation of such work we are indebted to Sherrington who analysed the phenomena in mammals and applied the results, in some detail, to the theory of co-ordinated locomotion.

Sherrington showed that when a hind limb of a spinal dog or cat responds by flexion to a suitable stimulus, the contralateral limb simultaneously displays an active state of extension: he also showed that a stimulus applied to the hind limb not infrequently involved co-ordinated changes in the posture of the fore limbs and vice versa (figure 10). In many tetrapods, normal ambulation is, in fact, characterized by simultaneous flexion of the left fore limb with the right hind limb (see figures 11 and 12, plates 4, 5), and during this process the limbs of the other diagonal are undergoing extension. This type of co-ordination could readily be achieved if flexor activity in the left fore limb automatically involved flexion of the right hind limb (and vice versa). So long as a rhythm were maintained in one limb, all four might be expected to step in a co-ordinated manner.

In 1910 Sherrington envisaged such a mechanism, but the existence of stepping in a deafferentated pair of antagonists tended to limit, in his view, the significance of the flexor reflex (and of its repercussions in other limbs) as an essential part of the locomotory mechanism. Recent work has, however, revived interest in this question, and it is pertinent to consider how far the flexion reflex so characteristic of a vertebrate limb can be shown to elicit, in the other three limbs, responses which simulate the phenomena of active locomotion.

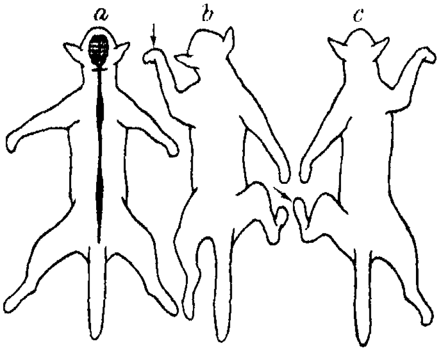


FIGURE 10. The reflex figure of a high spinal animal, showing the postures assumed on stimulating the extremities. Note the influence of the hind limb on the fore limb and vice versa. (From Fulton 1938.)

Before considering how far the behaviour of a spinal animal throws light on the nature of normal locomotion it is necessary to have a clear picture of the sequence of limb movements as seen in the intact animal. These movements, although often highly characteristic of particular species of higher vertebrates, are often variable according to the degree of locomotory activity being displayed. For example, a horse may amble, step, trot, canter, or gallop; in each case the motion of one limb relative to the others changes in a definite manner. In lower terrestrial tetrapods, however, the basic plan is usually clear and is shown in figures 11 and 12 (plates 4, 5). Starting with the flexion of a right fore limb, the sequence of flexor movements is as follows: (i) right fore limb, (ii) left hind limb, (iii) left fore limb, (iv) right hind limb. This series is particularly clear in slowly moving amphibia; as the speed of movement increases, the time interval between the two members of each diagonal pair of limbs decreases until the movement of both members is almost simultaneous. The absence of any further modifications in gait renders the newt or toad peculiarly suitable animals for study.

FLEXOR ACTIVITY IN THE STEP OF SPINAL AMPHIBIA

A frog or toad in which the spinal cord has been severed just posteriorly to the hind margin of the medulla never displays spontaneous walking

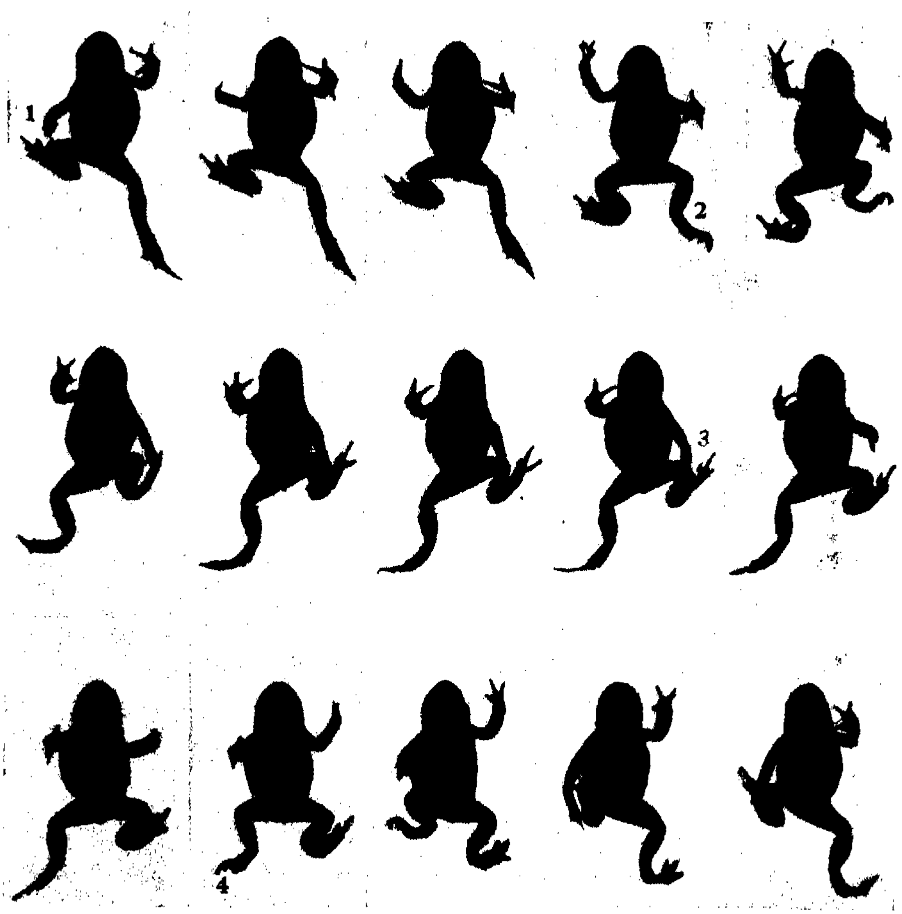


Figure 11. Normal pattern of limb movement in the toad (*Bufo*). The large numerals indicate the sequence of flexion of the limbs. 1, left fore; 2, right hind; 3, right fore; 4, left hind. Each of the fifteen photographs was taken after an interval of $\frac{1}{12}$ sec.

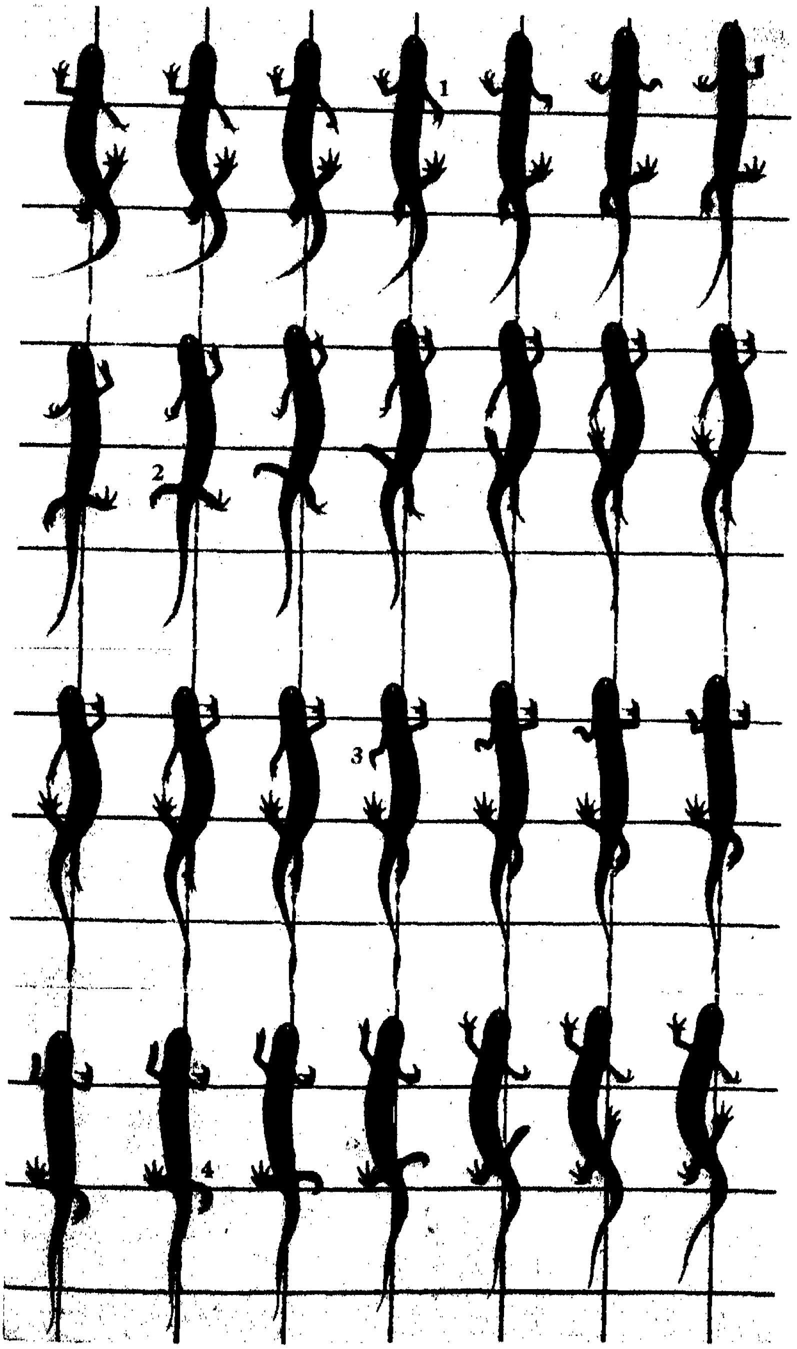


FIGURE 12. Normal sequence of limb movement in the newt (*Triton cristatus*); the numerals indicate the sequence of flexion. 1, right fore; 2, left hind; 3, left fore; 4, right hind. The photographs were taken at intervals of $\frac{1}{12}$ sec.

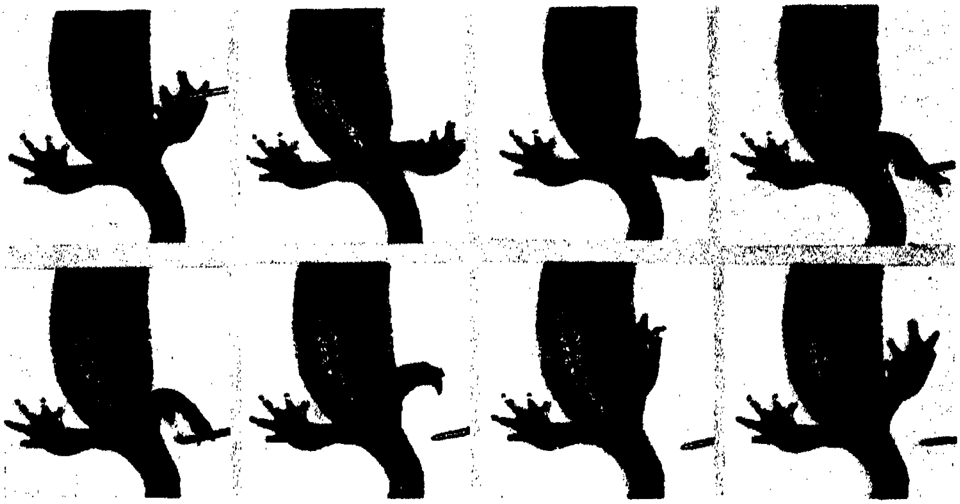


FIGURE 13. Flexor reflex in right hind limb of *Triton cristatus* induced by passive extension of the limb.

movements, but shortly after the operation each hind limb displays the well-known flexion reflex when subjected to a suitable stimulus. If the preparation be suspended vertically in air, both hind limbs are passively extended, but a limb quickly flexes if its toes are suitably stimulated. If, on the other hand, the posterior ventral surface of the abdomen is kept in contact with a solid surface (e.g. when the animal is lying on a table), the normal attitude of the limbs of the spinal animal is in the fully flexed position, and they resist any extraneous attempts to extend them by the operator. If, when the animal is at rest, a sliding platform is placed under one hind limb, so that it can be passively extended, no response occurs until the limb has reached a critical degree of passive extension, at which moment active flexion occurs. It is important to notice that when the limb is being extended by the moving platform, it is receiving a pattern of peripheral stimulation which is very similar to that which must arise during the normal extensor phase of the step of an intact animal. The reflex is at once abolished by severance of the dorsal roots of nerves 8, 9 and 10. Such excitation of flexor muscles in response to passive extension of a limb is of very widespread occurrence among vertebrates and arthopods. It occurs in mammals, amphibians, insects (ten Cate 1928) and myriapods, and we may therefore conclude that each limb is capable of generating the conditions necessary for activity in its flexor muscles.

In spinal preparations of the common newt (*Triton cristatus*) the flexor response to passive extension is of particular importance since it can be seen to be identical in every detail with the movement executed by the limb during the active flexor phase of normal locomotion (figure 13, plate 6).

When a normal amphibian is walking, the two hind limbs never flex simultaneously. It is, therefore, instructive to find an exactly similar phenomenon when a spinal toad, frog or newt (Snyder 1904; ten Cate 1928) is drawn passively over a level surface. Starting from rest, with both limbs flexed, the two hind limbs are passively extended together—but flexion never occurs simultaneously. Continuous traction produces a regular and persistent alternation of flexor movements which is clearly identical in many respects to normal ambulation and which does not readily fatigue. For a further study of these phenomena Dr Lissmann and I have employed high spinal preparations of toads or frogs in which flexor response to passive traction could be elicited from each of the four limbs. The animal was placed on the horizontal surface of a kymograph drum, and kept in position by forceps attached to a denervated area of skin on the roof of the head; rotation of the drum enabled passive traction to be applied to the limbs. When all four limbs of such a preparation are in contact with a moving surface, all four limbs retain their diagonal sequence of movement; when the fore limbs are supported on a stationary platform, the hind limbs being in contact with the drum, only the latter

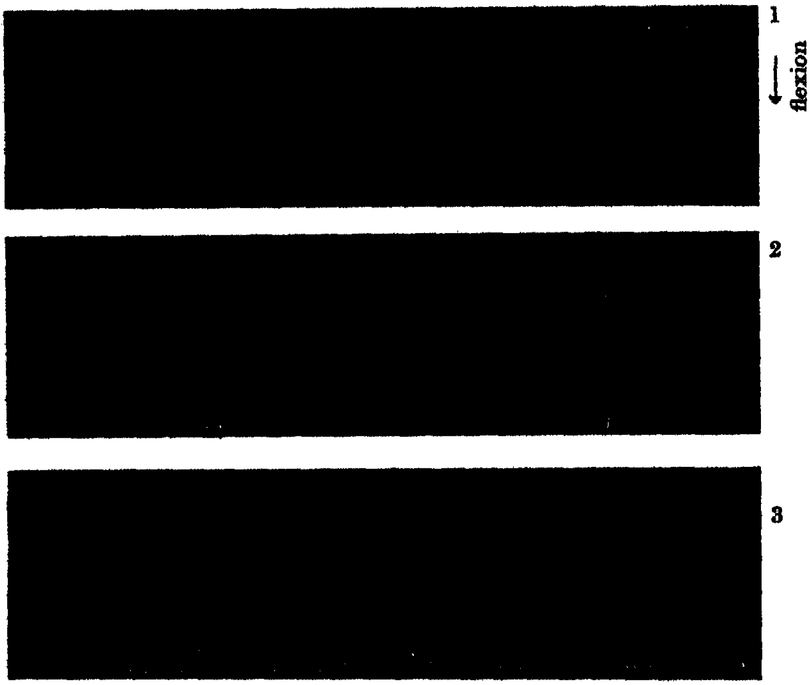


Figure 14. Record showing the effect of flexor reflexes in the fore limbs on the frequency of stepping of the hind limbs of a spinal toad. The preparation was mounted so that the hind limbs were always subjected to the drag of a moving drum, the fore limbs could be placed on or removed from the drum surface at will. In the top section of the tracing the fore limbs were not in contact with the drum—note the slow frequency of stepping of hind limbs: between the arrows in section 2 of the record, the fore limbs were in contact (*on*) with the drum and exhibited stepping movements—note increase in frequency of stepping of hind limbs. Subsequent removal (*off*) of fore limbs from the drum induced marked reduction in frequency of stepping of hind limbs; the frequency again rose when the fore limbs were replaced (*on*) on the drum. The three sections together form a continuous record and each section reads from right to left. *rp*, tracing of right posterior limb; *lp*, tracing of left posterior limb.

show any flexor activity and the frequency of their movements is markedly reduced (figure 14)—in two cases their movement ceased altogether. On lowering the fore limbs on to the drum they immediately begin to step and the frequency of flexion of the hind limbs at once increases. A closer study of these preparations revealed the following facts: (i) The response of a hind limb to flexion of the contralateral fore limb depends upon the posture of the hind limb at the moment of flexion of the fore limb: if this flexion occurs when the hind limb is only partially extended, the process of extension is either accelerated or replaced by flexion. If flexion of the fore limb occurs when the hind limb is nearly fully extended, flexion of the latter occurs immediately. Figure 15 illustrates this phenomenon. (ii) With the fore limbs stepping on the drum and the hind limbs lying on a smooth glass plate, active movements occurred in the hind limbs shortly after each movement of a fore limb. (iii) When the preparation was towed passively along the bench, the average length of step performed by a hind foot could be increased by 30 % by holding the fore limbs stationary on a fixed platform which moved with the body. (iv) In fatigued specimens, responses to movement of the fore limb only occurred in the hind limb at every alternate flexion of the former (see figure 15, B).

These results show clearly that a high level of co-ordination can exist between the flexor muscles of the four limbs without an equivalent display of phasic activity in the extensor muscles. Each limb, whilst capable of responding by flexion to passive extension, is, nevertheless, subject to influences exerted on it by flexor activity in the other three limbs; these influences are such as maintain, during rectilinear traction, the diagonal pattern of co-ordination typical of locomotion in an intact animal.

Precisely similar phenomena occur in the newt (*Triton cristatus*). If the spinal cord of a newt is cut at an appropriate level, spontaneous locomotion is completely and permanently abolished but each limb shows a marked flexion reflex on being passively moved to the fully extended position (figure 13). If such a preparation is placed on a suitably moving drum and one of the limbs only is in contact with the drum, that limb alone steps at a frequency depending on the speed of the drum's surface. If both hind limbs are on the drum they step alternately as in the case of the frog or toad. If all four limbs are placed on the drum they all step and exhibit, after a short preliminary period, the diagonal sequence so characteristic of normal locomotion.

So long as all four limbs of a spinal newt are subjected to passive rectilinear drag, either from a moving drum or by traction of the preparation over a suitable flat surface, the resultant stepping exhibits three characteristics: (i) there is a very regular alternation of movement of the fore limbs; (ii) there is a regular alternation of movement of the hind limbs; (iii) the diagonal pattern (RF, LH, LF, RH) is well defined. So far as can be seen, the spinal newt displays the full pattern of flexor activity exhibited by the normal animal—not only is the typical diagonal sequence retained

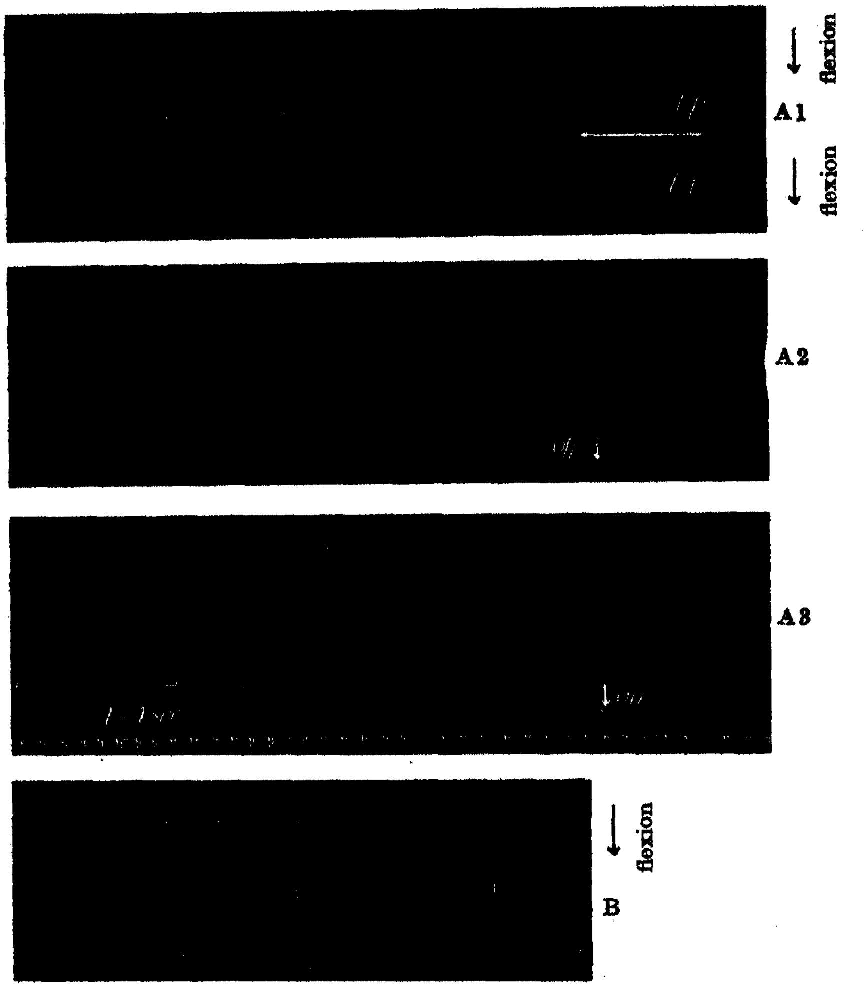


Figure 15. A 1–3. Three sections of a continuous record showing the effect of flexion of the left fore limb (*la*) of a toad on the activity of the right hind limb (*rp*). In section 1 all four limbs were in contact with the surface of a moving drum; note that flexion of the fore limb usually caused extension of the hind limb if the former occurred before passive extension of the hind limb was complete, but caused flexion of the hind limb if the passive extension of the hind limb was nearly complete. In section 2 of the record the fore limbs were removed (*off*) from the drum surface; note slowing of the rhythm of the hind limb. In section 3 the fore limbs were replaced (*on*) on the drum. Tracing B shows the response of a relatively fatigued preparation; note that the hind limb sometimes responded by flexion to every alternate flexion of the fore limb.

when the animal is moved passively along a straight line, but every detail of the movements executed by flexor muscles is preserved, the limb being lifted forward and placed down with characteristic precision.

Very different results are obtained if the animal is towed along the circumference of a circle. Under such conditions the number of steps taken is no longer the same for all four limbs; the normal diagonal sequence is absent and the number of steps taken in a given time by any one limb depends on the distance over which it is passively moved—as soon as a limb has been towed into the position of full extension it flexes.

	No. of steps performed by each limb in unit time			
Nature of traction	Left fore	Right hind	Right fore	Left hind
Straight line	16	15	16	16
Clockwise circle of 5 in. diameter	16	8	12	10
Clockwise circle of 3 in. diameter	16	0*	7	13

* The planta of the right hind limb remained at or near the centre of the circle.

The regularity and persistence with which co-ordinated flexor activity can be elicited from the limbs of a spinal frog, toad or newt makes it difficult to believe that the underlying mechanism plays no fundamental role in the locomotory activity of a normal animal. The spinal animal appears to retain a level of co-ordinated activity in its flexor centres identical with that of an intact animal, and it is significant that these centres continue to give their normal response although, as will now be shown, the corresponding extensor centres may be relatively inactive.

Extensor activity in the step of spinal Amphibia

Whereas a spinal newt when passively towed over the ground appears to give a relatively complete and persistent picture of the flexor activities seen during the movement of an intact animal, no trace of activity is usually seen in the extensor muscles of the limbs, and extraneous stimuli applied to one limb have, so far, failed to elicit, with any regularity, extensor activity in that or other limbs. In a few preparations, definite evidence of extensor activity has been observed, but it is difficult to produce such manifestations at will. The extensor muscles of nearly all preparations can, however, be excited by tactile stimulation applied to the tail. If a hind limb is passively flexed forward and the tail is then tapped slowly with a pencil or rubbed gently between the finger and thumb of the operator, the limb actively extends until it reaches the position of full extension, it then flexes forward and again extends if the stimulus to the tail be continued; a definite approximation to a complete stepping rhythm is thus obtained. If, before applying the stimulus to the tail, the limb be in a state of passive but not quite complete extension, stimulation of the tail induces flexion. The response of the limb to caudal stimulation thus depends upon its passive postural position at the moment of application of the stimulus. In this respect the muscles in the limb of a spinal newt clearly resemble those of the spinal dog (Magnus 1909, see p. 46).

A spinal frog or toad differs from a spinal newt by a display of a definite, although limited, degree of extensor activity. It is well known that a nociceptive stimulus which excites flexion in one hind limb of a frog simultaneously excites extension in the contralateral limb. There can be little doubt that this phenomenon throws some light on the mechanism of normal locomotion, but two points must be borne in mind: (i) the applied stimulus is usually different in kind to, and more intense than, that which is likely to arise naturally during normal locomotion; (ii) the effect of an extensor stimulus from the contralateral limb may under certain conditions be masked partially or entirely by the activity of ipsilateral reflex arcs tending to maintain flexion. In order to avoid these difficulties to some extent, Dr Lissmann and I have recently attempted to explore the extensor repercussions of a flexor reflex by using toads in which three limbs have been completely deafferentated, and in which the ventral roots of the fourth limb, the left posterior, have been cut. The posterior hind limb is therefore sensitive but makes no muscular response to stimulation; the other three limbs can respond to stimuli reaching them from the left posterior limb but cannot themselves initiate sensory activity. Figure 16 shows that a stimulus (capable of inducing flexion in an intact left posterior limb) applied to the de-efferentated left posterior limb causes extension in the contralateral deafferentated hind limb, a state of passive extension remaining in this contralateral limb after the effect of the stimulus has passed; there is no sign of subsequent flexion. When this experiment is repeated on a preparation in which the sensory roots of the right posterior limb have not been cut, the initial extension of the right hind limb (consequent to stimulation of the left de-efferentated hind limb) is rapidly replaced by active flexion (see figure 17). Thus a normal limb, which responds by extension to an extraneous stimulus, subsequently flexes by virtue of stimuli generated in its own proprioceptor sense organs. Just as a well-defined crossed extensor reflex exists in the hind limbs of a toad, so, by similar methods, a comparable mechanism can be shown to exist between a fore limb and an ipsilateral hind limb (figures 18 and 19).

FIGURE 16. Crossed extensor reflex in right posterior deafferentated limb (*rp*) of *Bufo* in response to tactile stimulation of de-efferentated left posterior limb (*lp*): both fore limbs were deafferentated. Note that extension of the right posterior limb is not followed by flexion.

FIGURE 17. Crossed extensor reflex in right posterior intact limb (*rp*) of *Bufo* in response to tactile stimulation of de-efferentated left posterior limb (*lp*). Both fore limbs were deafferentated. Note that initial extension of right posterior limb is followed by flexion.

FIGURE 18. Response of deafferentated right posterior limb of *Bufo* to tactile stimulation applied to de-efferentated left posterior (*lp*) or de-efferentated left anterior limb (*la*). On stimulating left posterior limb the right posterior limb extends; on stimulating left anterior limb the right posterior limb flexes.

These observations are closely parallel to those made by Sherrington (1910) on the intact limbs of a high spinal cat where a nociceptive stimulus applied to one limb, and therein causing flexion, induced extension both in its contralateral fellow and in the other ipsilateral limb (figure 10). It is important to remember, however, that although crossed extensor reflexes are of widespread occurrence among different groups of vertebrates, they usually only display themselves when a contralateral limb is exposed to a somewhat violent stimulus. It is, therefore, necessary to consider how far they can be elicited in response to the type of stimulus likely to arise during normal locomotion. Using spinal frogs and toads it is possible, under favourable conditions, to show that active extension is induced in an ipsilateral hind limb when normal flexion occurs in a fore limb, but it is not sufficiently powerful to propel the body forwards. In an intact toad, extensor repercussions of a primary flexion reflex have also been observed; figure 20, plate 7, shows that passive extension of an intact right fore limb, causing a flexor response in that limb, simultaneously elicits (i) extension in a deafferentated ipsilateral hind limb and (ii) flexion in a deafferentated contralateral hind limb.

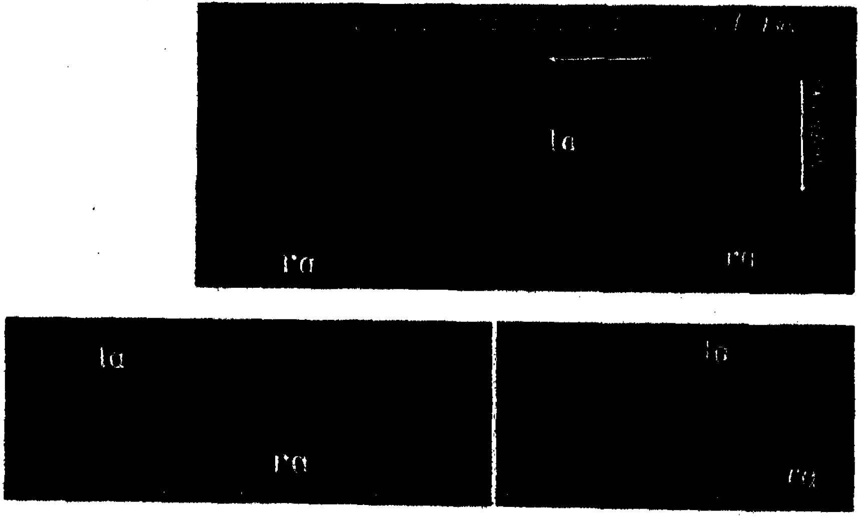


FIGURE 19. Response of deafferentated left posterior limb of *Bufo* to tactile stimulation applied to de-efferentated right anterior limb (*ra*) or left anterior limb (*la*). On stimulating left anterior limb the left posterior limb extends; on stimulating the right anterior limb the left posterior limb flexes but this is sometimes followed by extension.

It may be noted that something, approximating to normal locomotion,

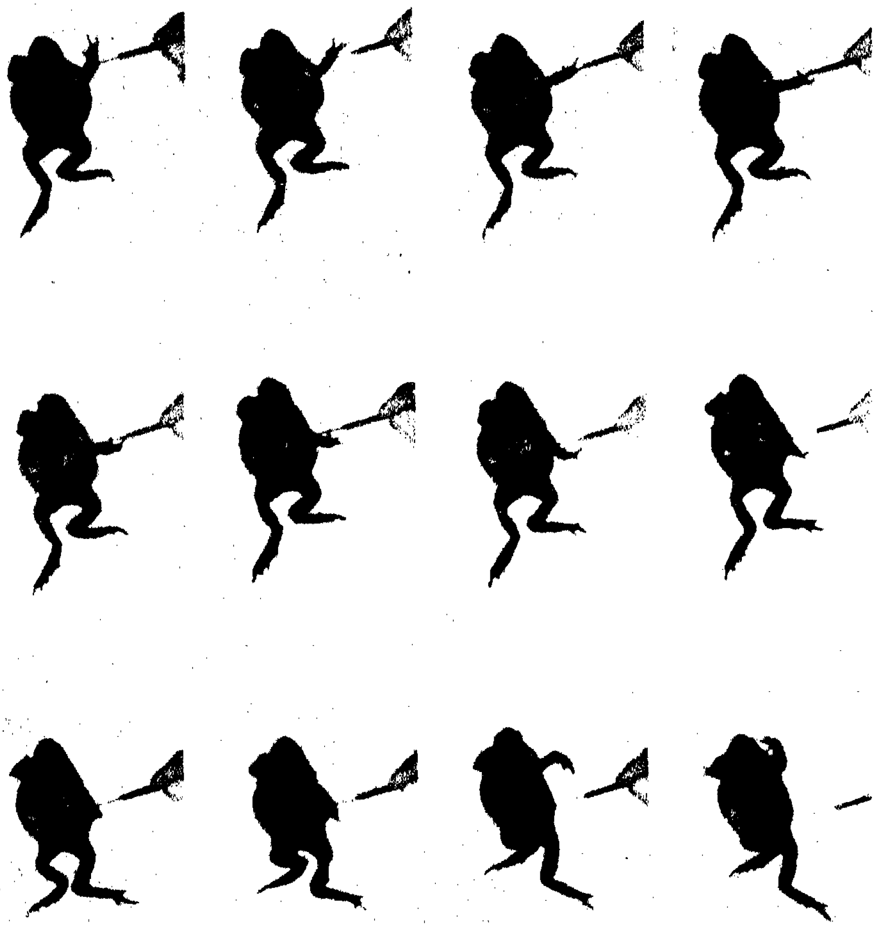


FIGURE 20. Response of deafferentated hind limbs of a toad (*Bufo*) to passive extension of intact right fore limb. The right fore limb itself flexes, the left hind limb also flexes, and the right hind limb extends. This preparation showed no response in the left fore limb; other, similar, preparations not infrequently exhibited extension of this limb.

seems to emerge when we superimpose this picture of secondary extensor reflexes on that already provided by a study of the flexor activities of a spinal newt or toad. We are tempted to conclude that a spinal toad or newt would walk actively were it not for the fact that the intensity of stimulation emerging from the dorsal roots of a flexing limb is insufficient to reach the threshold required to excite the extensor centres of the other limbs concerned.

Any attempts to give a rigid analysis of the locomotory mechanism of the amphibia is clearly premature, but a working hypothesis might, perhaps, be available along the following lines. Let us assume that all four limbs are lying passively in the positions characteristic of full flexion. From actual observation we know that an extrinsic tactile stimulus will induce simultaneous activity in the extensor muscles of all four limbs; as each of the four limbs approaches the position of full extension, its own proprioceptor activity will excite its flexor centres and inhibit its extensor centres. Let the intensity of this proprioceptor stimulation first reach, in the right fore limb, the intensity required to dominate the primary extensor activity. The following events will then occur. (i) The extensors of the right fore limb will be inhibited and the flexors will contract. (ii) The flexor centres of the left hind limb (which are already being excited by proprioceptor activity in the limb itself) will receive additional excitation from the right fore limb, and will therefore induce flexion of the left hind limb (see figure 20). (iii) Flexor activity in the left fore limb and right hind limb will be inhibited and their extensor muscles will remain active. As soon as the right fore and left hind limbs have reached the extended position, the other two limbs will be free to flex by their own proprioceptive mechanism, and these flexions will in turn induce extensor activity in the right fore and left hind limbs. Since the fore limbs tend to excite the hind limbs more than vice versa, a diagonal sequence of stepping will occur and will be maintained.

The essential feature of the above hypothesis lies in the suggestion that the change over from extensor to flexor activity is dependent on proprioceptor activity in at least a limited amount of the active musculature; the co-ordination of the four limbs is due to the influence exerted by the sensory centres in one limb on the activity of the motor centres of the other three.

Little, if any, originality can be claimed for this hypothesis; it is essentially the same as that put forward by Sherrington (1910) for mammals prior to the discovery of stepping in a pair of deafferentated antagonistic muscles. We thus seem to reach an impasse—we must either believe that the observations of Sherrington and Graham Brown are of the nature of locomotory artefacts or that the co-ordination of peripheral reflexes seen in the amphibia has no fundamental bearing on the normal locomotory function. Only further work can resolve this difficulty. For the moment, however, it seems legitimate to recall that the only locomotory rhythms known to occur in intact terrestrial animals whose limbs are deprived of external stimulation are those characteristic of swimming and not of ambulation. A totally deafferentated frog can swim actively. Is it possible that a swimming rhythm may be centrally controlled, whilst peripheral events control terrestrial ambulation?

LOCOMOTION IN GROUPS OTHER THAN TETRAPOD VERTEBRATES

Perhaps the most important conclusion to be drawn from a study of locomotion in the lower tetrapod vertebrates is that a limited region of normally active locomotory musculature can impress upon the whole central nervous system a pattern of activity in its motor centres which will enable other desensitized muscles to display their normal co-ordinated movements. So long as part of the neuromuscular mechanism is intact, other muscles can take up their correct phase relationships to each other; a change in the activity of any one intact muscle automatically involves a change in those of all the others. It is of interest to consider how far this concept can be applied to other types of locomotion.

The primitive pattern of vertebrate locomotion is the undulatory type exhibited by fish and many aquatic invertebrates; it is characterized by the passage of muscular waves passing alternately down each side of the body. Similarly, in many terrestrial forms locomotion is effected by the passage of muscular waves over a series of appendages (polychaet worms, myriapods, etc.) or over the surface of the whole or part of the body (earthworm, snail). It is generally assumed that co-ordination of this type is due to the passage of excitatory stimuli down the nerve cord at a rate identical to that of the resultant muscular wave (see Coghill 1929). In typical teleostean fishes and in larval amphibia this concept is perhaps feasible, but when applied more widely it encounters difficulties. (i) Some fish (e.g. the freshwater eel, Gray 1936) and nearly all snakes exhibit very clearly the phenomenon of "standing" waves; the body can remain at rest in a typical undulatory posture for relatively long periods of time. (ii) The rate at which the acquisition of the undulatory pattern spreads over the limbs of a myriapod or over the body of *Nereis diversicolor* is very much greater than the velocity of the subsequent muscular waves

(Gray 1939). Similarly, in the snail (*Helix aspersa*) the wave pattern spreads posteriorly over the surface of the foot at a rate which is greater than that at which the muscular waves travel forwards; in this case also "standing" waves can be observed.

Some, at least, of these difficulties are overcome if the theory of excitatory waves passing down the nerve cord is replaced by the concept of reflex patterns spreading through the nervous system at a rate which is independent of the time required for each muscle to complete one complete cycle of locomotory movement. This view is supported by a study of the behaviour of the spinal eel (Gray 1936). If a point near the tail of a spinal eel be gently stimulated, localized ipsilateral contraction occurs; if the stimulus be more intense, a secondary region of contraction develops anteriorly and contralaterally to the first (figure 21); if the stimulus be sustained, these two regions of contraction pass posteriorly along the body and the animal swims. It is clear that two phenomena are involved: (i) the development of a reflex pattern; (ii) the development of a rhythm in all the muscles concerned. The development of the reflex pattern is obviously a phenomenon possessing much in common with the spread of reflex patterns over the limbs of a tetrapod.

When we consider such forms as the myriapod or *Nereis* (Gray 1939), the inadequacy of the "excitatory wave" theory becomes even more obvious. In these forms the muscular waves pass anteriorly along the body; the existence of an excitatory centre at the posterior end of the body is excluded by the fact that no disturbance is caused by amputating the posterior end of the body. In these cases, the movement of any one limb is clearly not dependent on the arrival of a wave of excitation which has successively initiated similar muscular movements in all limbs lying posteriorly to itself. In both *Nereis* and *Doratogonus*, the onset of locomotion occurs by a very rapid spread of pattern over the whole body starting at the head end of the animal (e.g. every eighth or tenth appendage exhibits almost simultaneous movements in phase with each other); only then, and at a much slower rate does the pattern move forwards over the body. Precisely similar phenomena are observed in the snail: movement starts by the spread of a pattern of contraction and relaxation over the whole foot, and is followed by a movement of this pattern, at a slower rate, over the surface of the foot. In all these cases the ambulatory pattern develops very rapidly and independently of the frequency of the subsequent rhythm.

From a functional standpoint, an animal such as a myriapod with an inextensible body must, when starting from rest, actuate its anterior limbs

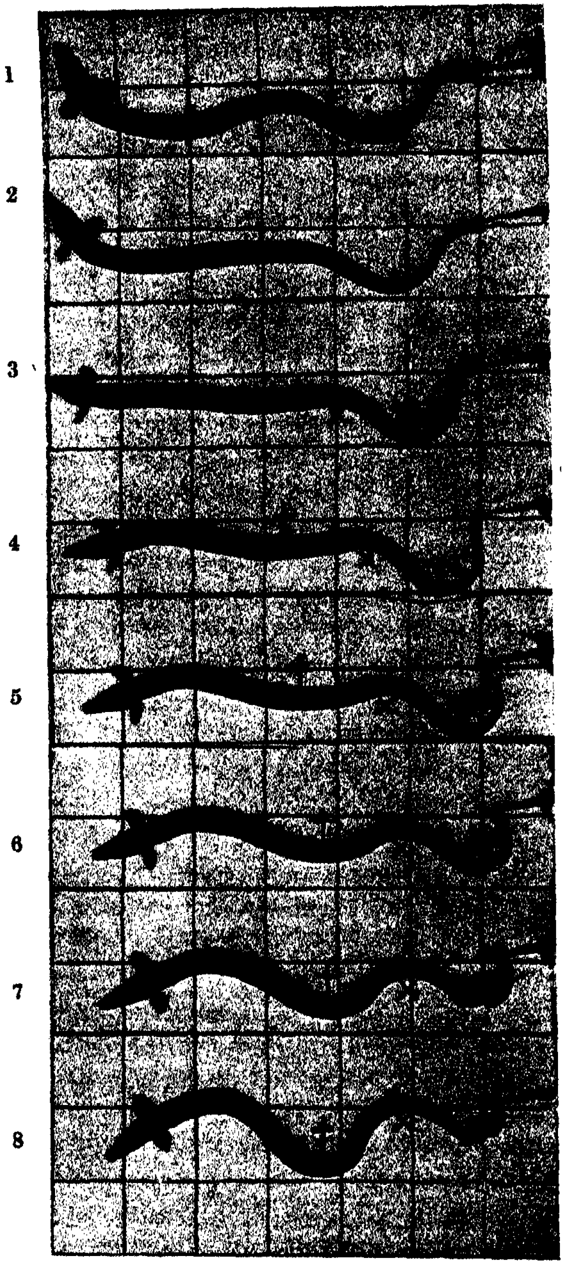


FIGURE 21. The response of a spinal eel to a persistent stimulus applied to the tip of the tail. Note the formation and movement of active waves of contraction indicated by the symbols •, ×, +. (From Gray 1936.)

in less time than is required for the passage of a muscular wave over the whole length of the body.

SUMMARY

The available facts appear to establish four main points concerning the mechanism of locomotory co-ordination: (i) To a surprising degree it is independent of the higher nervous centres, although in vertebrate animals the integrity of the medulla oblongata and, sometimes, its contiguous regions, is essential for a display of full locomotory activity. (ii) The type of locomotion displayed by an animal often varies with the pattern of peripheral stimulation to which it is exposed. (iii) Few, if any, animals display co-ordinated motion when their central nervous systems are completely isolated from those patterns of peripheral stimulation which reach them under conditions of normal progression. It is often, however, possible to restrict the receipt of this peripheral stimulation to a comparatively small amount of the total musculature taking part in subsequent locomotion. (iv) The response made by spinal (or comparable) preparations to appropriate patterns of peripheral stimuli seems to justify the view that each phase of a normal ambulatory rhythm is initiated and controlled by peripheral events.

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Twin research from the time of Francis Galton to the present-day

By Professor Dr Freiherr von Verschuer, *Frankfort-on-Main*

(*Lecture delivered before the Royal Society on* 8 *June* 1939)

In the course of the exchange of professors between the Royal Society and the Kaiser Wilhelm-Gesellschaft zur Förderung der Wissenschaften, I have received the honoured invitation for this lecture. I feel bound to express my thanks to you, Mr President. I accepted the invitation with great pleasure, and have seized the opportunity to cultivate personal relations with our English colleagues and to further the connexion between English and German science.

In the library of the University Institute for Human Genetics and Eugenics, founded and led by me, there hangs a single picture, that of Francis Galton. We honour in him the founder of our science. It was quite a special pleasure for me to visit to-day the famous Galton Laboratory and to make the acquaintance of the present successor of Galton, Professor Fisher. The connexion with the Galton Laboratory, which I have always cultivated, has thus become closer and more personal.

"The exceedingly close resemblance attributed to twins has been the subject of many novels and plays, and most persons have felt a desire to know upon what basis of truth those works of fiction may rest. But twins have a special claim upon our attention; it is, that their history affords means of distinguishing between the effects of tendencies received at birth and of those that were imposed by the special circumstances of their after-lives."

These are the introductory sentences of the paper written by Galton in 1875: "The history of twins as a criterion of the relative powers of nature and nurture." In 1883 Galton occupied himself once more with the problem of twins in his book *Inquiries into human faculty and its development*. He was looking for, in his own words, a "new method by which it would be possible to weigh in just scales the effects of Nature and Nurture, and to ascertain their respective shares in framing the disposition and intellectual ability of men". Galton was right in stating: "I have not found any book that treats of twins from my present point of view". He thus became the founder of twin research, which, together with and alongside research into family history, constitutes the most important method of research into human heredity. The investigation of twins was for Galton, too, only a part of the science of the transmission of hereditary characteristics in man and of its application in eugenics.

Galton traced the life and career of pairs of twins and described them in interesting detail. In a number of cases he found extraordinary resemblances in physical and mental characteristics. In another group of twins, different from birth, identical environment and education had no power to produce resemblance. He thus arrives at the conviction that innate characteristics are of essential importance for man's development.

Galton's ideas were not followed up with further research work until 1905 by Thorndike in America, and by Poll in Germany in 1914. In medical literature there are of course to be found accounts of observations on individual pairs of twins who attracted attention by the similar nature and development of their illnesses. The first comprehensive statements were made by Apert in 1923 and Siemens in 1924. Since then the methodology of twin research has been further developed, and twin research has led to the most valuable results in the investigation of the hereditary nature of individual characteristics. Hundreds, even thousands, of scientific papers from almost every civilized country bear witness of this.

For those amongst the audience who have not such a close acquaintance with the problems of human genetics, the following may be noted. Man's heredity was first investigated by means of research into family stocks. Analogies were established to the Mendelian Law. This method is, however, only applicable when we have a relatively simple and regular rule of heredity. Nature has presented us with the second important method, twin research. Its application is based on the fact that there are two sorts of twins: (1) one-egged twins, who arise from the division of an originally unified embryo, and (2) two-egged twins, who arise from the simultaneous fertilization of two ova by two spermatozoa. The former are alike in their whole hereditary disposition. The latter are in their heredity as alike as ordinary brothers and sisters: they are thus always different in a part of their heredity. By the comparison of the two groups of twins the share of heredity and environment as causes for the development of human qualities may be ascertained.

Twins have long played a part in medicine as a special case of pregnancy. Scientists learned to distinguish twins born in a common foetal membrane and those born in separate ones. The first were thought to be monozygotic or one-egged, the latter dizygotic or two-egged. I shall show at once that this assumption of gynaecology is no longer correct in this form. The researcher into heredity requires a method to enable him to establish from the twins themselves whether they are monozygotic or dizygotic, that is to say, quite independently of any data at hand relating to foetal membrane placenta at birth. To-day we make general use of diagnosis by similarity. This has been especially developed as a methodology by the experience of the last 15 years. We compare in the partners of a pair numerous characteristics which we know to be predominantly determined by heredity. Each hereditary characteristic may be employed which occurs, in the population from which the twins come, in various genotypes. The colour of eyes and hair, for instance, are characteristics which vary greatly amongst Europeans and are therefore valuable for us in the diagnosis of twins. The same characteristics cannot, however, be employed in the case of the universally dark-eyed and dark-haired Mongols and Negroes, because here monozygotic as well as dizygotic twins always have the same colour of eyes and hair. The most suitable characteristics are those which appear nearly always alike in one-egged twins and, on the other hand, frequently unlike in two-egged twins. A high degree of susceptibility to environment renders a characteristic useless for this diagnosis. Blood groups, colour of eyes and hair, for instance, do not vary at all, or only very slightly, under the influence of environment. But blood groups and type of hair are found to be alike in more than half the cases of two-egged twins. Thus these characteristics cannot be applied in the diagnosis, whereas the colour of eyes and hair, as well as the form of the ear, are more rarely alike in two-egged twins and are therefore more frequently applicable as tests. As soon as a characteristic (such as, for instance, the folds of the tongue, the capillaries of the skin and the papillary lines of the fingers) does not often vary even in the case of one-egged twins, its value for the diagnosis of twins is diminished.

The reliability of the diagnosis by similarity I have checked by serological experiments. Each pair of twins was diagnosed on the basis of morphological comparison as monozygotic or dizygotic. Blood tests were carried out in a laboratory quite independent of us for the blood groups O, A, B and AB, and for the serological qualities M and N. In many hundreds of cases of monozygotic twins, not one case occurred exhibiting any serological difference. Even if very similar dizygotic twins were to be found amongst our monozygotic twins, they would show partly different blood groups. Such a case did not occur. This proves the correctness of our twin diagnosis. We were able to prove further that the frequency of similar and dissimilar blood groups amongst our dizygotic twins completely corresponded to the results to be expected from pairs of ordinary brothers and sisters. If, by mistake, we had classed dissimilar monozygotic twins in the group of dizygotics, we should have expected a greater similarity in the blood groups than in the case of twins of different sexes of pairs of brothers and sisters. Thus this finding also confirms the reliability of our diagnosis by similarity. I have therefore no hesitation in taking it as the solid foundation of our research.

The next thing was to compare the diagnosis by similarity and the postnatal data with one another. It was soon shown that the data provided by the daybooks of women's clinics do not form a reliable basis. Together with my colleagues Curtius, Lassen and Steiner, I collected 10 years ago, in Berlin, the placenta of newborn twins and examined them minutely. The placenta vessels were filled with an opaque contrast fluid, and a stereo-X-ray plate of the vessel tree was made. The septum between the two foetal cavities was tested by microscopic inspection for the presence of two amnea and two choria, or for two amnea alone. Dichorial twin placenta always have separate circulations. In monochorial twin placenta vessels anastomoses are always to be found. Diagnosis by similarity was first applied, in repeated examinations, to the twins, in so far as they survived, at the age of 6 months at the earliest. A compilation of our findings is given in table 1. The 32 monochorial pairs of twins are all, according to expectation, one-egged. Of the 100 dichorial pairs, however, only 76 are two-egged, as was the earlier expectation, whilst 24 are one-egged. Thus of the 56 one-egged pairs, 32 are monochorial and 24 dichorial. Accordingly, it is quite a frequent phenomenon that one-egged twins should be born in separate foetal membranes.

TABLE 1

Post-natal data		Diagnosis by similarity	
Monochorial	Dichorial	Monozygotic	Dizygotic
32	—	32	—
—	100	24	76
32	24	56	—
—	76	—	76

How can this result be explained? We know that the fertilization of the ovum, that is, the union of sperm and ovum, occurs in the tube. The implantation of the germ in the mucous membrane of the uterus occurs only on the 8th to 10th day after fertilization, at the stage of the blastula or commencing gastrulation. We must assume that the splitting of the germ, which leads to the formation of one-egged twins, occurs very early in some cases. The division leads then to two completely separate germs which can also implant themselves, quite separate from each other, in the mucous membrane of the uterus. As a consequence of this separate implantation of the two halves of the germ the placenta can also be completely separated; we were able to establish this in 16 out of the 24 dichorial one-egged pairs.

For the application of twin research as the method of hereditary biology there is yet another question of particular importance: are one-egged twins hereditarily alike? Through unequal division of the cell or through the mutation of the genes after this division, gene differences could occur between one-egged twins. Yet, in practice, we need not reckon with such an eventuality. We have no basis for assuming that gene differences occur in one-egged twins. The hereditary equality of one-egged twins can be supported by three different proofs.

(1) In the case of differences between the twins, one proves that they are not determined by heredity. Such a proof is provisionally impossible in the case of man. It was applied by Kappert to flax plants. In the flax plant, *Linum usitatissimum*, one-egged twins, triplets and quadruplets occur in certain stocks. Phaenotypic differences between the pairs of twins and triplets were shown when cultivated by self-pollenization not to be hereditary, that is, to be conditioned by environment.

(2) The test of the diagnosis by similarity, by the methods of serological comparison already mentioned, reveals at the same time the important

fact that, in a large number of one-egged twins, *not once* was a difference found in certain hereditary characteristics; in practice, therefore, it is not necessary to reckon with gene differences in one-egged twins.

(3) The third proof is based on the comparison of the similarity of the two one-egged twins with the similarity of the two halves of the body. Numerous investigations have revealed that in chance variations of characteristics the similarity between the one-egged twins is no greater than that between the right and left half of the body of one person. In the characteristics, however, which show a typical right-left variation, the homologous halves of the body in one-egged twins are more alike than the right and left sides of one person.

Thus it may be taken as proved that monozygotic twins are really identical beings. It is not, therefore, the case that the one twin is a "regenerated" right half of the body, and the other a completion of the originally left determinant as was often assumed. Mirror-image resemblance also is not a regular phenomenon in monozygotic twins. The division into embryonal determinants happens before the differentiation of right and left halves of the body. The two twins thus born resemble each other not only in the hereditary determinants but also in normal asymmetric relations. All differences between one-egged partners are non-hereditary.

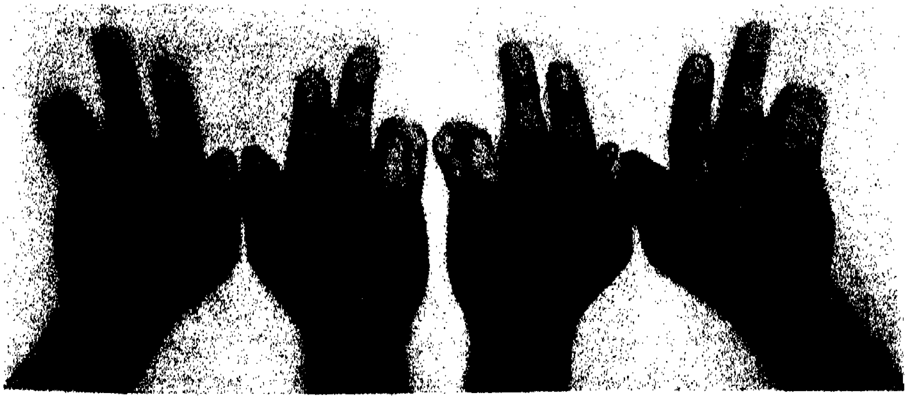


FIGURE 1. Four hands of a pair of monozygotic twins with syndactylism and contraction (after K. H. Bauer).

The significance of the observation of twins for research into heredity may be shown now by specific data with the use of slides. In this picture (figure 1) are represented the four hands of a pair of one-egged twins. The syndactylism and contraction of the third and fourth finger of each hand exhibit a similarity, a good example of the exact and regular manifestation of a gene. This case is especially interesting for another reason. The syndactylism was separated by an operation, and the wound covered by grafting from one twin to the other. Whereas graftings from one human to another do not normally grow, in this case it did. One-egged twins behave here in this important reaction test as a single person.

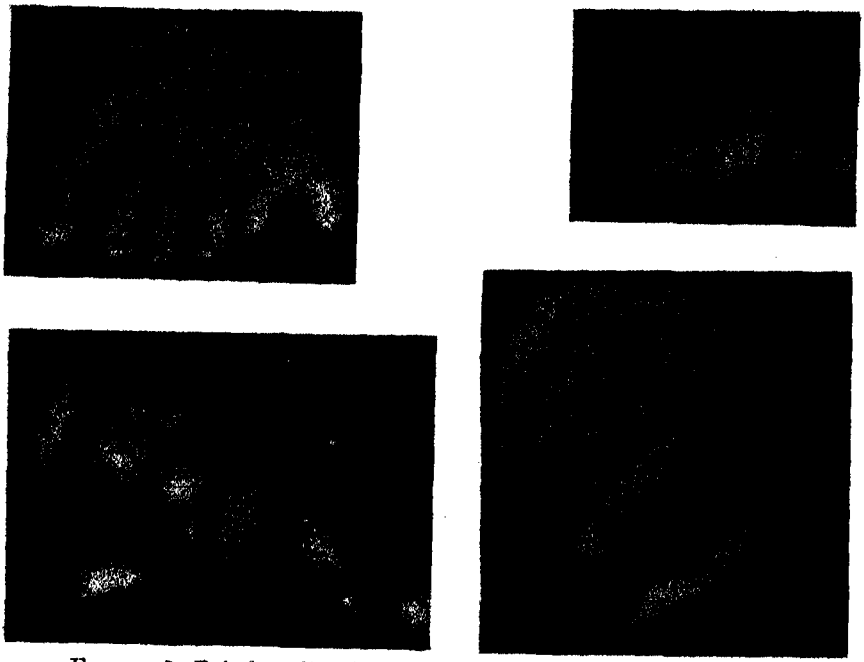


FIGURE. 2. Polydactylism in monozygotic twins (after W. Lehmann and E. A. Witteler).

In figure 2 we have a case of polydactylism in one-egged twins. The one hand shows a complete doubling of the little finger. The corresponding hand of the other twin shows merely an appendage with two small nuclei of bone and a nail. On the third hand, at the edge of the little finger, there is a wart-shaped protuberance only; the fourth hand is normal. Of the four feet, only one shows a doubling of the little toe. The other feet exhibit no variations. In this case it is a question of a dominant hereditary characteristic. The gene, however, reveals an imperfect power of penetration. Of the eight extremities only three show marked anomalies. The form of expression of the gene is also not constant. It varies between complete doubling of the little finger or the little toe and a small, barely visible protuberance of the skin.

A very typical arrested malformation is known as hare-lip and cleft-palate. One pair of monozygotic twins shows the anomaly in mirror-image resemblance. In another case of monozygotic twins, described by Claussen, only one of the pair shows a cleavage of lips, jaw and palate. In the other this diseased gene has led only to an underdevelopment of one of the upper side incisors, which is clearly smaller and more pointed than on the other side. The same gene produced in both twins a defective heart (innate cardiac defect) and led to sexual underdevelopment. Both are impotent and the genital organs are too small. These defects are more marked in the case of the one with the cloven palate than in that of his twin.

The hereditary nature of ability can be very clearly shown by means of data about twins. An interesting case is that of the twin brothers Piccard. Their resemblance in youth and as students was quite extraordinary. Their scientific interests were the same, but finally August applied himself to physics and Jean to chemistry. August became a professor in Brussels and Jean a professor in an American University. Shortly after August had made the first flight into the stratosphere, Jean ventured the same voyage in America. Since then the two brothers have taken turns in making successful and unsuccessful flights.

There is a very interesting case in the history of the Bach family. The father of Johann Sebastian had an identical twin. It is reported of the two: They were so alike that even their own wives couldn't distinguish them. Language and thought were quite alike. They played in the same way, and they conceived their recitals in the same way. If one were ill, the other was too. One died very soon after the other. A similar case of the present day I was able to observe: The conductors Wolf and Will Heinz were both pupils of Max Reger. They then lived for 20 years apart from each other as conductors of different orchestras. They told me, *inter alia*, of the following incident: During an opera production Will conducted the first act. After the interval Wolf relieved him without anyone in the audience or even a single member of the orchestra noticing the change of conductor. I succeeded in putting on record the completely similar movements of the two musicians when conducting by means of a film.

However interesting such individual results are, they do not yet permit of any more general conclusions. For that, the compiling and statistical treatment of a large body of data is necessary. Permit me to give a short account of the research into tuberculous twins which I have been carrying on for more than 10 years with my friend Karl Diehl and which we published in book form in 1933 and 1936. Investigation into the question of the hereditary factor in tuberculosis is extremely difficult owing to the manifold nature of the operative causes, and has not been completely solved by previous methods. We undertook a systematic search for pairs of twins in which at least one partner was tuberculous. Through the co-operation of hospitals, sanatoria and tuberculosis welfare centres who asked each patient whether he were a twin, we succeeded in finding over two hundred pairs and subjecting them to repeated examination. The establishment of likeness and unlikeness of a tubercular growth is extraordinarily difficult. A single discovery is not enough. The development of the disease must be ascertained by years of observations and by repeated detailed clinical and X-ray examinations.

Of 80 monozygotic pairs, 52 pairs, that is 65%, show a similar and 28 pairs a dissimilar reaction to tuberculosis. Of 125 dizygotic pairs, however, only 31 pairs (that is 25%) were found with similar and 94 (75%) with dissimilar reactions.

With the numbers available the difference between the frequencies of 65 and 25% is amply significant. The discrepancy from proportionality, χ^2, is 32·7166, or 31·0695 when adjusted for continuity by Yates's correction. The latter value corresponds with a probability of only one in 40,000,000 of obtaining so great a disparity by chance.

This marked difference between the two groups of twins can only be explained by the effects of heredity. By means of careful investigations we followed up all possible environmental influences. In the case of monozygotic twins with tubercular symptoms only in one partner, particular environmental influences could always be proved to be the determining factors of the disease. But in contrast to these cases we find a group of monozygotic twins who, in almost complete independence of all environmental influence and partly in direct defiance of differences in environment, show an extremely high degree of similarity in the development of the disease in each of the twins. The more frequent and greater discordance of tubercular symptoms in dizygotic twins could, however, only be explained in a few cases by the effects of environmental factors. The cause of this dissimilarity is rather to be seen in the difference of heredity in the twins.

I should like to supplement these general statements by showing a few individual cases. In figure 3 are shown the X-ray photographs of monozygotic twin sisters, 26 years old; they have been living apart for 9 years, the one as a shop girl in Berlin, the other as a needlewoman in the country in East Prussia. Both fell ill fairly suddenly at the same time with high fever and the typical symptoms. The second twin, the Berlin one, came quickly to hospital and a pneumothorax was applied. In twin no. 1, however, tuberculosis was only discovered 3 months later as a consequence

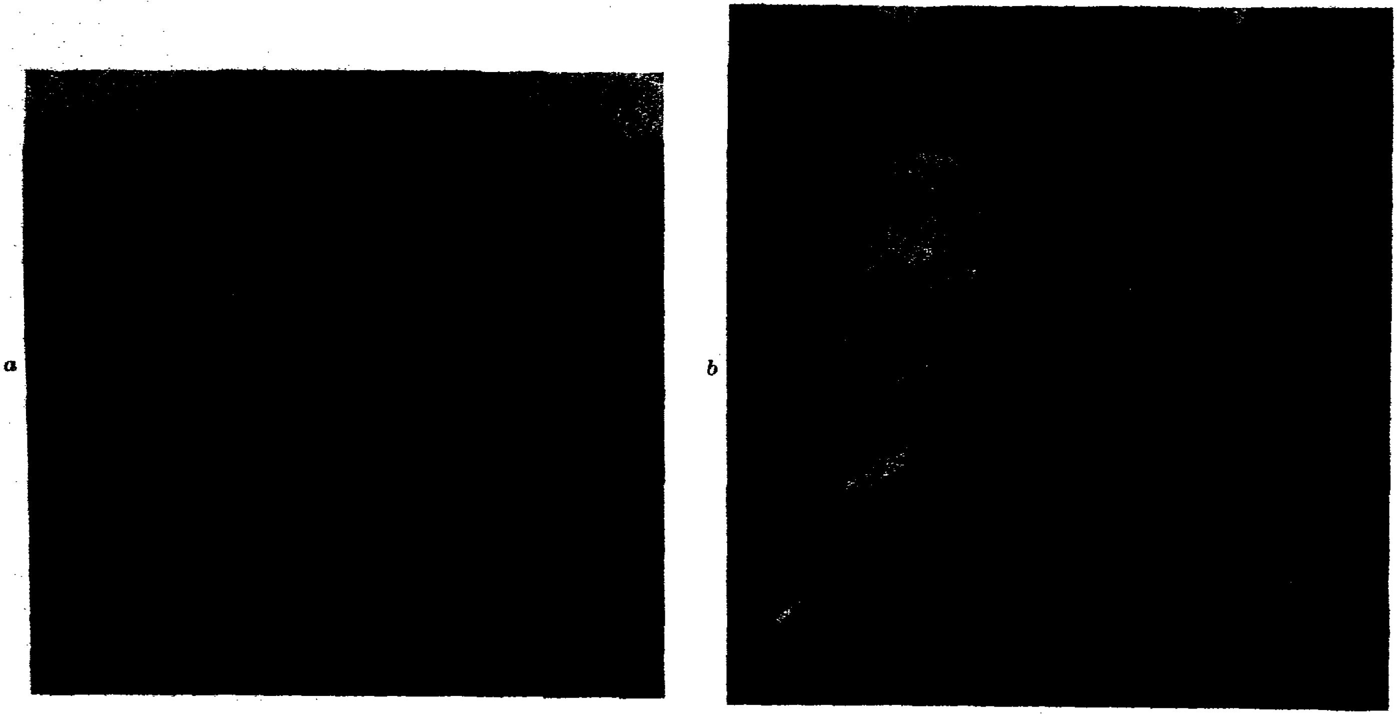


FIGURE 3. Monozygotic twins with similar tuberculosis (after K. Diehl and O. v. Verschuer). (*a*) Twin no. 1, 15 April 1930. Extensive tuberculosis on the left side with cavity under the clavicle. Scattered, predominantly productive foci on the right, especially towards the apex. (*b*) Twin no. 2, 2 January 1930. Large infiltration in the left upper lobe, with large foci near and also in the middle of the right lung. Cavity not definitely demonstrable by X-ray, but ascertained clinically.

of our twin research. The X-ray shows a highly similar condition, more advanced in twin no. 1. Both have on the left, under the clavicle, a great cavity. It was not possible to secure medical treatment for no. 1, and she died 15 months later. Twin no. 2, on the other hand, always had the best attention, yet she only lived 5 months longer.

In the next figure (figure 4) are shown X-ray photographs of 25 years old monozygotic brothers. Both were locksmiths by trade and had been living apart for 9 years. Twin no. 2 fell ill with tuberculosis of the right lung. He underwent sanatorium treatment and a pneumothorax was applied. Twin no. 1 was invited to an examination. Hitherto he knew nothing of the disease. The X-ray shows old and new spots in the left apex and a circular infiltration on the right. He also was treated but declined the application of a pneumothorax. The next figure (figure 5), 18 months later, shows no. 2 after seropneumothorax and a large new infiltration in the left apex. In twin no. 1 the infiltration on the right has subsided, but in the left lung the process has gone still farther. Other X-ray photographs were made 3 years later (figure 6). Twin no. 2 is passably well, the infiltration in the left apex has receded. Twin no. 1 shows extensive disease in both lungs. He died a few months later. Twin no. 2 is still alive but a gigantic cavity has developed in his left lung, so that he is left with only a very small remnant of lung for breathing. His decease is close at hand. Thus, despite many differences, we find in both twins the disease taking a highly similar course.

The photographs in figure 7 are of monozygotic twin brothers of whom twin no. 1 developed tuberculosis of the calcaneus. Four years later his brother had tuberculosis in precisely the same spot. In view of the infrequency of the disease in this spot, we cannot assume this to be a pure coincidence.

The following case shows something very interesting. One of a pair of monozygotic twin sisters developed tuberculosis of the lungs. She underwent treatment a year later, the X-ray showing both lungs highly diseased. Great cavities were found on the left below the clavicle and in the middle of the right lung. The patient died 5 months later. The twin sister now submitted to medical supervision, the first X-ray showing merely a loosening of the left hilus with increased cord formation at the top on the left. Six weeks later the disease is clearly to be seen on the spot where, in the sister, the great cavity was. A pneumothorax was applied on the left. Six months later, however, signs of the disease are also to be seen in the middle of the right lung, once more on the same spot as in the twin. A pneumothorax was also applied on the right and the disease was successfully cured. This twin was, without a doubt, saved by timely treatment.

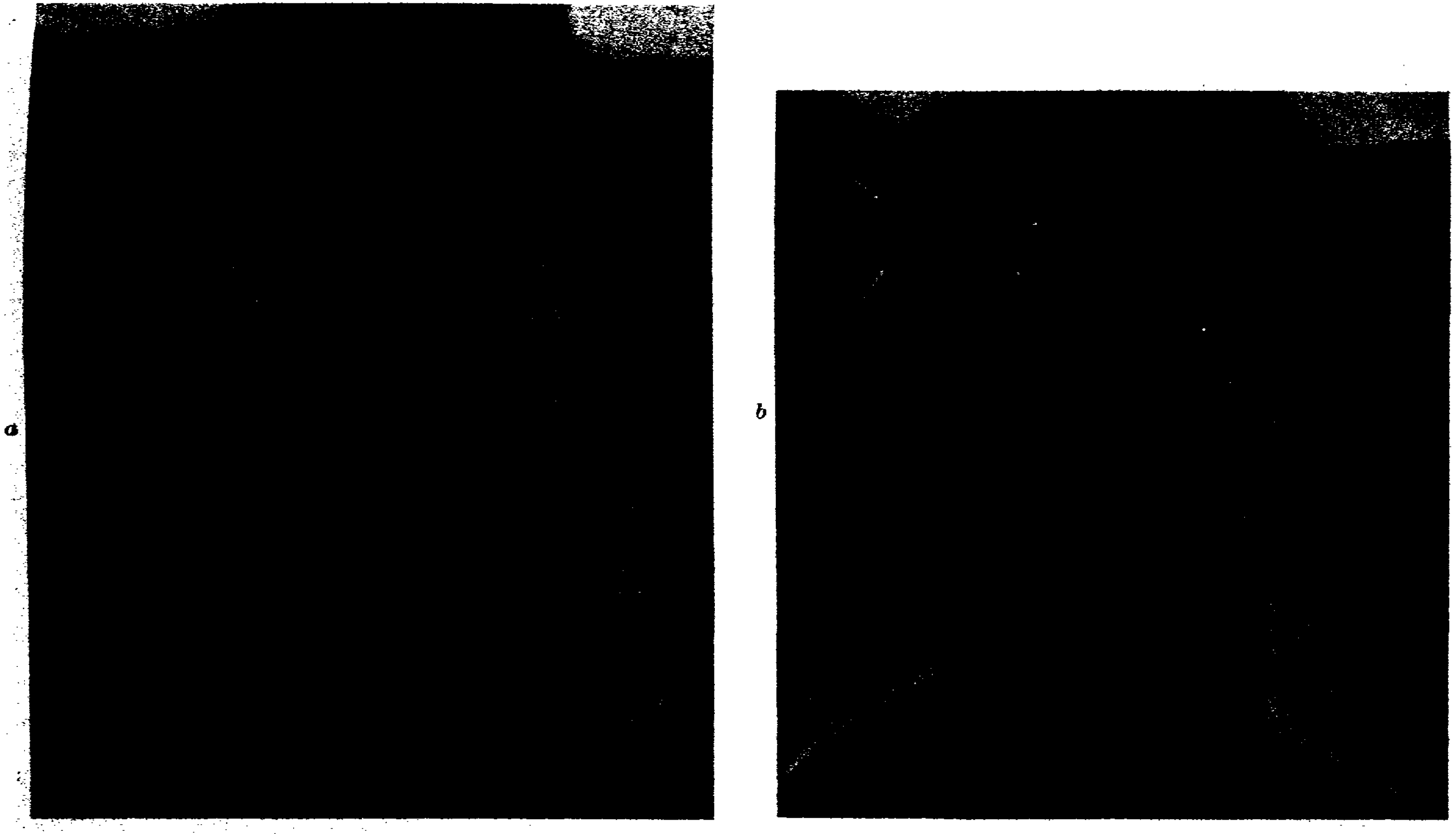


FIGURE 4. X-ray photographs of monozygotic twin brothers (after K. Diehl and O. v. Verschuer). (*a*) Twin no. 1, 24 November 1929. Tuberculosis of the upper lobe on the left with circular infiltration on the right in the middle. The foci at the top on the left are partly soft and mottled, and partly calcified. (*b*) Twin no. 2, 12 June 1929. Large tuberculosis cavity in the right upper lobe. Its outline is shown by arrows. In the remaining upper lobe tissue are scattered thickly crusted dark patches.

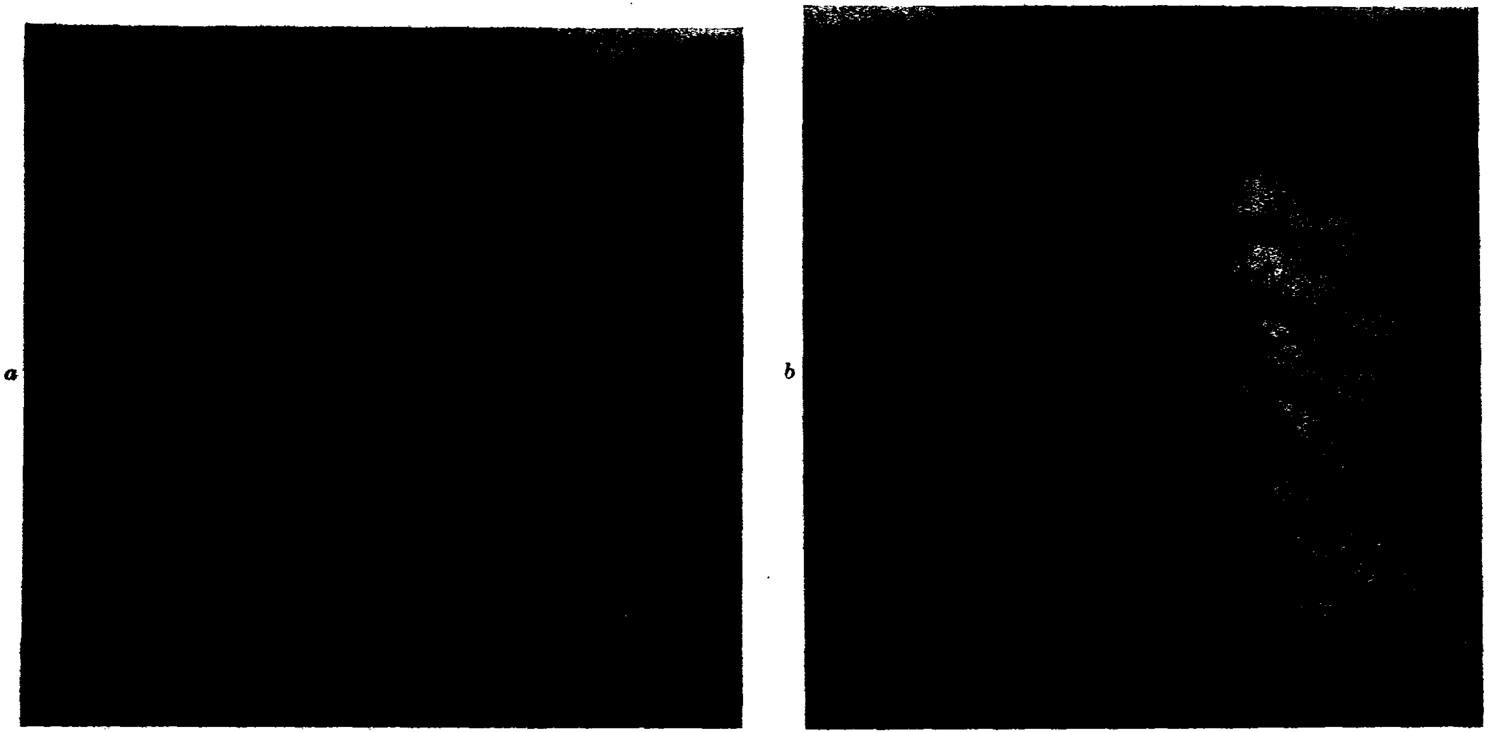


Figure 5. X-ray photographs of the same twin pair as figure 4 (after K. Diehl and O. v. Verschuer). (*a*) Twin no. 1, 5 February 1931. Commencing resolution in the left upper lobe. Round focus on the right in the middle has gone. (*b*) Twin no. 2, 5 February 1931. Condition after seropneumothorax on the right. Advancing tuberculosis in the upper lobe on the left.

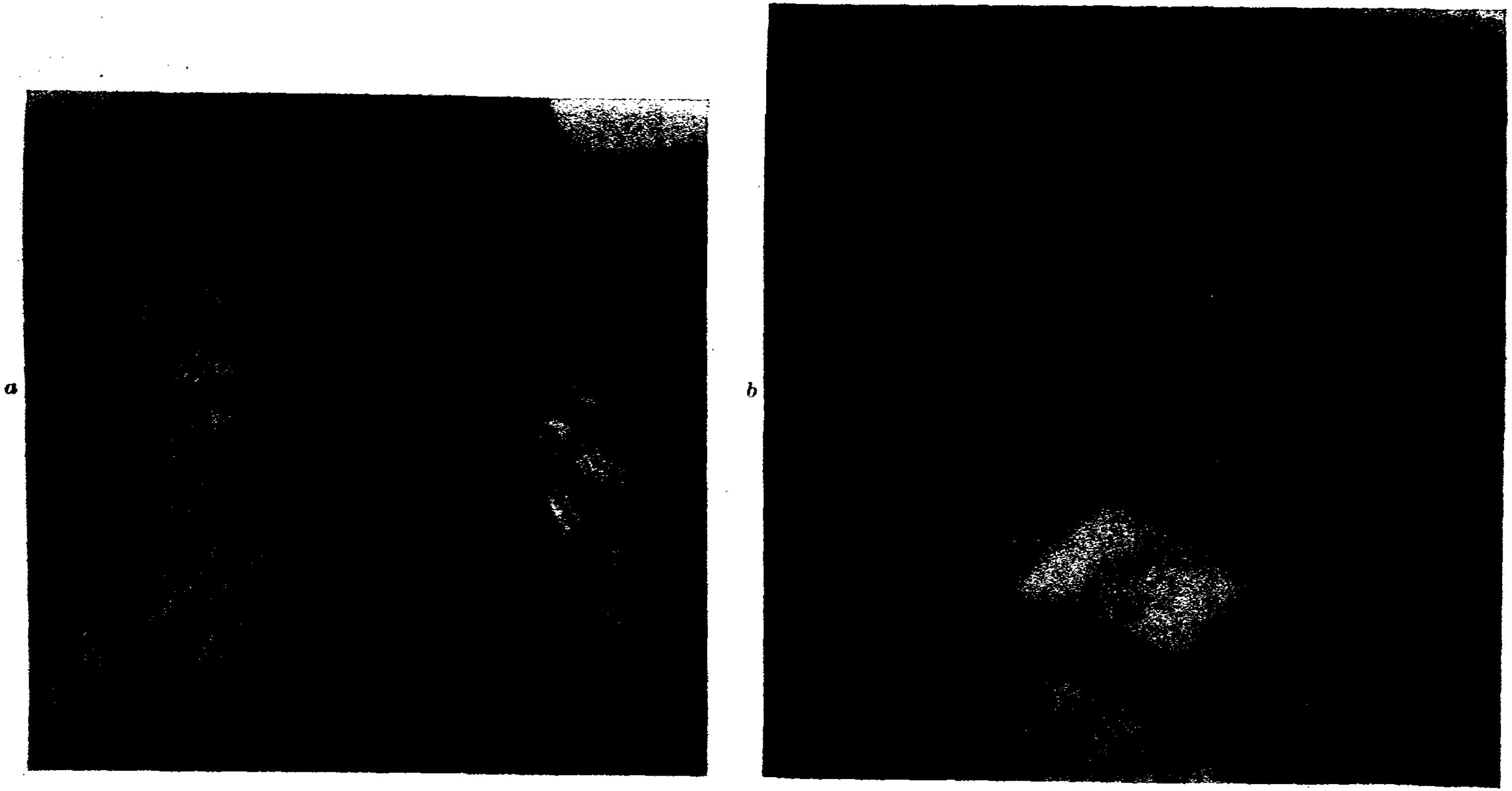
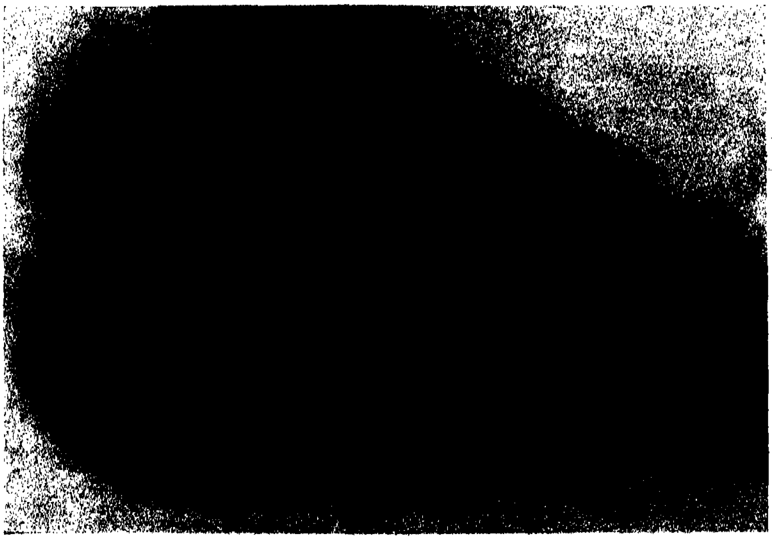


FIGURE 6. X-ray photographs of the same twin pair as figures 4 and 5 (after K. Diehl and O. v. Verschuer). (*a*) Twin no. 1, 17 January 1934. Large cavity in the left upper lobe with acinous-nodose foci in all the other lobes. (*b*) Twin no. 2, 17 January 1934. A section shown life-size: left apex and upper zone. The earlier infiltration in the left upper part is cirrhotically-productive.

a



b

FIGURE 7. Monozygotic twin brothers with tuberculosis of the calcaneus (after K. Diehl and O. v. Verschuer). (*a*) Twin no. 1, May 1925. Tuberculosis on the left. (*b*) Twin no. 1, August 1931. Condition after excochleation.

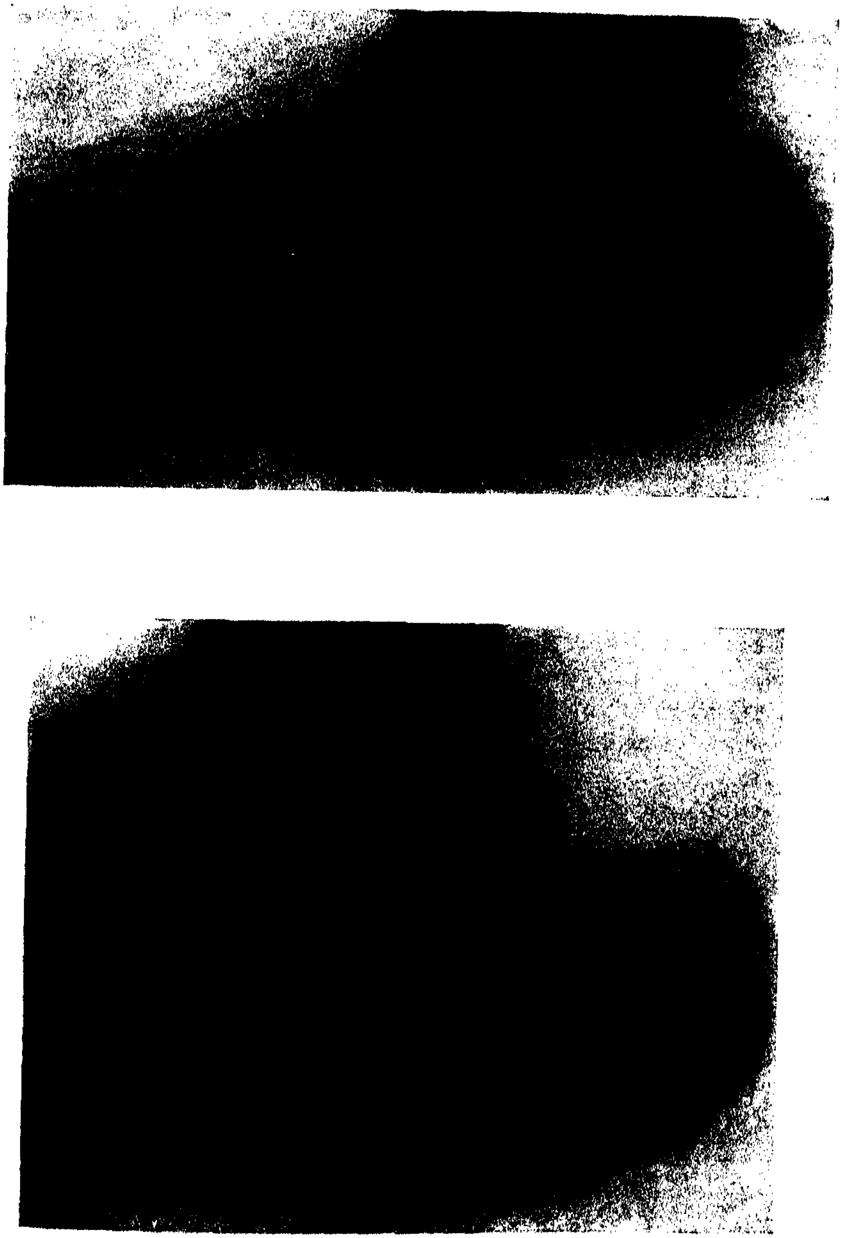


Figure 7. (*c*) Twin no. 2, April 1929. Tuberculosis of the calcaneus on the right. (*d*) Twin no. 2, August 1929.

In particular, pairs of twins with tubercular symptoms only in one of the pair require our attention. Out of 67 pairs of dizygotic twins of this sort, the diseased twin in one-third of the cases either died of tuberculosis, is still ill or has been cured. Amongst the 16 monozygotic pairs in this group, the diseased twin only died in one case. In 11 cases the tuberculosis was cured in the diseased partner. This frequent curing in the case of discordant monozygotic twins is due obviously to a hereditary power of resistance, which reveals itself in the health of the other partner.

From our investigations, which can only be shown in a few aspects here, we concluded that there exists a specific hereditary tendency to tuberculosis. This produces a more than average danger of developing tuberculosis and a lessened resistance during its course. The hereditary conditioning of tuberculosis is seen, above all, in the special nature and development of this disease. The lives and case histories of tuberculous twins provide adequate testimony of this.

In twin research the demand for unbiased series, free from the suspicion of selection, has been realized. As a very simple method, I proposed and put into practice, first in Berlin and then in Frankfort, the following: Each patient received in a hospital is asked whether he was born a twin or not. The answer is noted on a form. The partner of each twin thus discovered is also invited for examination. If he is dead, we attempt to throw light on the diagnosis by similarity, on the question of concordance and discordance even posthumously by collecting reports, photographs, and documents relating to illness. Thus we arrive without particular difficulty at scientifically usable data about the twins. However, monozygotic twins are discovered more frequently than dizygotic ones in this way, since adult dizygotic twins are very often no longer aware of their twin relationship.

An excellent method of acquiring a statistically impeccable series of twins is given by Luxenburger and applied by him for the first time in schizophrenia cases. From the reception lists and case histories of asylums and hospitals the name, date and place of birth of the person concerned—the bearers of the relevant disease—are secured. By reference back to the local registry or church records it is ascertained whether the people concerned were born twins or not. A great amount of preliminary data is necessary to secure an adequate series of twins, for a twin occurs roughly only once in every 60–80 people questioned, and only every second or third of the adult twins has a partner still alive. When one further considers that only every fourth pair of twins are monozygotic, it is easily understood that a great deal of work is necessary to secure valuable scientific data. Let us take an example from our research: In my institute Claussen and

Steiner undertook an investigation into twins who suffered from rheumatic diseases. 13,000 extracts were made from the records of rheumatism spas and sent with an answer form to the registry offices. 12,600 answers came back of which 11,000 could be used. Amongst these there were 127 twins, that is one twin to every 86 inquiries. 84 pairs of twins were of the same sex; both twins were living in 43 cases.

A further way towards achieving a representative series of twins is the compilation of all the twins within a defined area or length of time. With the aid of the registry offices all the twins are first discovered and then examined. Such a piece of research was carried out at my institute for the municipal area of Frankfort. For the years 1898–1937 altogether 4170 twin births were discovered in the registers of the Statistical Office. Further investigations showed that in 1911 pairs both partners were still alive.

We owe the latest development of the methodology of twin research to Gottschaldt, the director of the hereditary psychology department of the Kaiser Wilhelm-Institute of Anthropology in Berlin-Dahlem. It is the so-called "twins camps". Twin children of Berlin and other towns who were in need of a holiday were brought together in two camps, for a 6 weeks' summer holiday. Several doctors, psychologists, and trained nurses lived with the children during this time, kept them under constant observation, and took notes. Every experience of the twins from early morning until bedtime, the daily rhythm of life, the succession of moods, the reaction to difficulties, to successful and unsuccessful experiences and to conflicts were exactly noted. This kind of observation was supplemented by experimental psychological tests introduced unobtrusively into the daily life. These investigations have led to essentially new conclusions about the hereditary nature and development of normal psychological tendencies, about which we cannot give a detailed account here.

I should like to mention further, in conclusion, the research into monozygotic pairs of twins separated soon after birth or in early childhood and who grow up in a different environment. Newman, of the University of Chicago, has earned our thanks by the collection of very interesting material for research. It is precisely through these observations that, alongside the influences of environment and education, the significance of the hereditary determinant is most clearly seen. All these investigations hark back to the brilliant idea of Galton, which he expressed simply and clearly in the following way:

"Many a person has amused himself by throwing bits of stick into a tiny brook and watching their progress; how they are arrested, first by one chance obstacle, then by another; and again, how their onward course is facilitated by a combination of circumstances. He might ascribe much importance to each of these events, and think how largely the destiny of the stick had been governed by a series of trifling accidents. Nevertheless all the sticks succeed in passing down the current, and in the long run, they travel at nearly the same rate. So it is with life, in respect to the several accidents which seem to have had a great effect upon our careers. The one element that varies in different individuals, but is constant in each of them, is the natural tendency; it corresponds to the current in the stream, and inevitably asserts itself."

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The lipochromes and sterols of the algal classes

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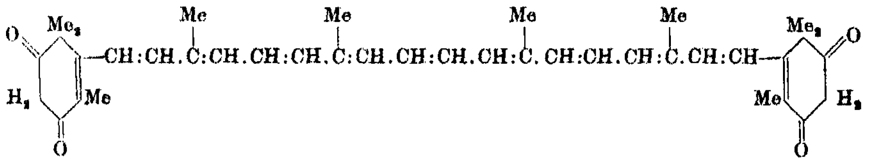
INTRODUCTION

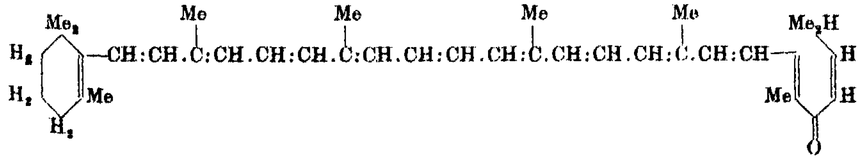
The lipoid constituents of the phanerogams, especially the sterols and carotenoid pigments, have for many years been the subject of intensive study, and the detailed knowledge of them which we now possess contrasts strikingly with lack of precise information concerning the occurrence and character of similar substances among the algae. That the algae contain sterols was first recorded by Willstätter and Page (1914), who noted the existence of a new phytosterol in the brown alga *Fucus vesiculosus*. Montignie (1935) has also recorded the presence of steroid material in certain algae. The first detailed investigation of algal sterols was carried out by Heilbron, Phipers and Wright (1934), who isolated from both *Fucus vesiculosus* and *Pelvetia canaliculata* a doubly unsaturated sterol of the formula $C_{29}H_{48}O$, to which the name fucosterol was assigned. This sterol is thus isomeric with stigmasterol, the phytosterol of soya and calabar beans (Windaus and Hauth 1906) and with ostreasterol, the characteristic sterol of oysters (Bergmann 1934). In a further paper Heilbron, Parry and Phipers (1935) showed that fucosterol is also present in the red alga *Rhodymenia palmata* and that it coexists with sitosterol in certain members of the Chlorophyceae.

The algal lipochromes have long been the subject of research, but this has, however, been mostly of a disconnected and empiric nature (cf. Boresch 1932). A wide survey of the field was made by Kylin (1927) who, although not isolating any pigments in the pure crystalline state, reached the conclusion that, apart from carotene which is ubiquitous and common to all classes, each algal class has associated with it its own characteristic lipochrome. The first application to the analysis of algal lipochromes of the modern methods of phase separation and chromatographic resolution developed by Kuhn and Brockmann (1932*a*) was described by Heilbron and Phipers (1935). These authors showed that whereas members of the Chlorophyceae contain β-carotene, lutein and possibly taraxanthin, in the Phaeophyceae the last two pigments are replaced by fucoxanthin (cf. Willstätter and Page 1914). Further application of these methods has revealed that the so-called "haematochrome" of the Trentepohliae (Cohn 1867; Zopf 1895) consists mainly of β-carotene (Heilbron, Lythgoe and Phipers 1935), a result since confirmed by Tischer (1936*a*). This author has also examined the haematochrome of red Euglenae, and has isolated from it as main constituent a substance of the formula $C_{40}H_{48}O_4$ which he has shown to be 4:6:4′:6′-tetraketo-β-carotene (I), isomeric with the crustacean pigment astacene (Karrer and Loewe 1934):



(I)

The new pigment, for which the name "euglenarhodone" was proposed (Tischer 1936*b*), has also been isolated from *Haematococcus pluvialis* where it forms the main lipochrome constituent (Tischer 1937). Heilbron and Lythgoe (1936) have further ascertained that the blue-green algae (Myxophyceae) contain a monoketone $C_{40}H_{54}O$ (II) to which the name myxoxanthin has been given:*



(II)

In continuation of these investigations we have now undertaken the analysis of a number of algae (see table 1) carefully selected from seven of the eleven classes known to British algologists (Fritsch 1935). As far as possible representatives of the main orders of each class have been investigated, as it was felt that this would reveal whether any relationship existed between the botanical classification and the appearance of specific pigments and sterols. In the classes Rhodophyceae and Phaeophyceae, where the more primitive orders of a particular class may perhaps be indicated from botanical evidence, these have been analysed in an attempt to determine whether the primitive or retrogressive tendencies were influenced by the lipoid constituents or vice versa. Particular care has been taken to ensure that only fresh material should be used (cf. Heilbron and Phipers 1935), and that it should be free from symbiotic and contaminating algae. The importance of the latter precaution was clearly demonstrated by Kylin (1927) who was unable to obtain *Ceramium rubrum* in a state uncontaminated by diatoms. The majority of the specimens examined have been collected from the shores of Cardigan Bay, where the growth of marine algae is free from contamination by ocean traffic. As a preliminary to the detailed examination of any alga, it was washed repeatedly with water, and only specimens free from all except the minutest trace of epiphytic diatoms were employed. The fresh-water algae were similarly cleaned and scrutinized. The more readily accessible algae, e.g. *Chondrus crispus* and *Gigartina stellata*, were examined in quantities of up to 10 kg. (wet weight), but the difficulty of obtaining supplies of the rarer algae, e.g. *Botrydium granulatum*, rendered it impossible either to employ comparable quantities of such species or to repeat the analysis at different seasons of the year. It must be emphasized, therefore, that where only relatively small amounts of a species were available, it is quite possible that substances present only in traces may have escaped detection; in all possible cases, however, where unexpected results were obtained, two distinct batches of material were examined.

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* Tischer (1938) has recently described the isolation from the blue-green alga *Aphanizomenon flos-aquae* of three new carotenoid pigments, which are obviously closely related to those described by Heilbron and Lythgoe (1936).

TABLE 1. LIST OF SPECIES EXAMINED WITH HABITAT

A. CHLOROPHYCEAE

Order	Family	Genus and species	Habitat
Ulotrichales	Ulvaceae	*Ulva lactuca* Linn.	Rock pools between tide-marks, Aberystwyth
		Enteromorpha compressa Grev.	Rocks at mid-tide level, Aberystwyth
Cladophorales	Cladophoraceae	*Cladophora Sauteri* (Nees). Kütz	Hickling Broad, Norfolk
Chaetophorales	Trentepohliaceae	*Trentepohlia aurea* Mart.	Walls at Ponterwyd, etc. Aberystwyth
	Pleurococcaceae	*Pleurococcus Naegelii* Chod.	Bark of trees; palings, Welshpool
Oedogoniales	Oedogoniaceae	*Oedogonium*	Aberdeen
Conjugales	Zygnemaceae	*Zygnema pectinatum* (Vauch) Ag.	Fresh-water pool, Whittington, Salop
Siphonales	Vaucheriaceae	*Vaucheria hamata* Lyngb.	Fresh-water stream, Aberystwyth
Charales	Characeae	*Nitella opaca* Ag.	Aberdeen

TABLE 1 (*continued*)

Order	Family	Genus and species	Habitat
B. XANTHOPHYCEAE			
Heterosiphonales	Botrydiaceae	*Botrydium granulatum* (L.) Grev.	Muddy surround of pool, Aberystwyth
C. BACILLARIOPHYCEAE			
Pennales	Nitzschiaceae	*Nitzschia closterium* (Ehr.) W. Smith	Marine, Aberdeen
D. CHRYSOPHYCEAE			
Chrysomonadales	Chrysocapsaceae	*Apistonema Carteri* Anand.	Chalk cliffs, Westgate-on-Sea
		Thallochrysis litoralis Anand.	
		Gloeochrysis maritima Anand.	
E. PHAEOPHYCEAE			
(1) *Isogeneratae*			
Ectocarpales	Ectocarpaceae	*Pilayella littoralis* Kjellm.	Epiphytic on Fucaceae, Aberystwyth
		Ectocarpus tomentosus Lyngb.	Epiphytic on *Fucus*, Aberystwyth
Sphacelariales	Sphacelariaceae	*Sphacelaria cirrhosa* C. A. Agardh. var. *pennata* Hauck.	Epiphytic on *Ceramium rubrum*, Aberystwyth
	Stypocaulaceae	*Stypocaulon scoparium* Kütz.	Submerged rocks between tide-marks, Aberystwyth
	Cladostephaceae	*Cladostephus spongiosus* J. G. Agardh.	Rocks between tide-marks, Aberystwyth
Dictyotales	Dictyotaceae	*Dictyota dichotoma* Lamour	Rocks between tide-marks, Aberystwyth
(2) *Heterogeneratae* (Polystichineae)			
Laminariales	Chordaceae	*Chorda filum* Lamour	On rocks in sandy rift, Aberystwyth
	Laminariaceae	*Laminaria digitata* Lamour	Rocks at low-water mark, Aberdeen
(3) *Cyclosporeae*			
Fucales	Fucaceae	*Fucus ceranoides* Linn.	Mouth of R. Rheidol, Aberystwyth
		Fucus vesiculosus Linn.	Rocks between tide-marks, Aberdeen
		Ascophyllum nodosum Le Jol.	Mid-tide level, Aberystwyth
		Halidrys siliquosa Lyngb.	Rock pools, low-tide level, Aberystwyth

TABLE 1 (*continued*)

F. RHODOPHYCEAE

Order	Family	Genus and species	Habitat
(1) *Protoflorideae* Bangiales	Bangiaceae	*Porphyra umbilicalis* J. G. Agardh.	Covering rocks at high water-mark, Borth
(2) *Florideae* Nemalionales	Lemaniaceae	*Lemanea* (*Sacheria*) *mamillosa* Kütz.	Swift-flowing fresh-water stream Ponterwyd, Aberystwyth
Gelidiales	Gelidiaceae	*Gelidium corneum* Lamour	Rocks, low-tide level, Aberystwyth
Cryptonemiales	Dumontiaceae	*Dilsea edulis* Stackh.	Rocks, low-tide level, Aberystwyth
	Rhizophyllidaceae	*Polyides rotundus* Grev.	Rock pools, low-tide level, Aberystwyth
	Corallinaceae	*Corallina officinalis* Linn.	(*a*) Rock pools above mid-tide level, Aberystwyth (*b*) Low-water pools, Aberystwyth
Ceramiales	Rhodomelaceae	*Polysiphonia fastigiata* Grev.	Parasitic on *Ascophyllum nodosum*, mid-tide level, Aberystwyth
		Polysiphonia nigrescens Grev.	Upper to mid-tide level, Aberystwyth
	Ceramiaceae	*Ceramium rubrum* C. A. Agardh.	Rock pools, mid-tide zone, Aberystwyth
Gigartinales	Gigartinaceae	*Chondrus crispus* Lyngb.	Rocks, low-water mark, Aberystwyth
		Gigartina stellata Batt.	Rocks, low-water mark, Aberystwyth
		Phyllophora membranifolia J. G. Agardh.	Rocks, low-water mark, Aberystwyth
		Ahnfeltia plicata Fries.	Rocks, low-water mark, Aberystwyth
Rhodymeniales	Rhodymeniaceae	*Rhodymenia palmata* Grev.	Rocks, low-water mark, Aberystwyth
		Plocamium coccineum Lyngb.	Rocks, low-water mark, Aberystwyth

G. MYXOPHYCEAE

Order	Family	Genus and species	Habitat
Hormogonales	Oscillatoriaceae	*Oscillatoria rubescens* D.C.	Fresh-water reservoir, London
	Rivulariaceae	*Rivularia nitida* J. G. Agardh.	Mud banks of Leri estuary, Borth
		Rivularia atra Roth	Rocks, mid-tide level, Aberystwyth

EXPERIMENTAL

General methods of extraction. Extraction was effected by allowing the minced alga to stand in absolute methylated spirits for 2–3 days in airtight vessels filled with carbon dioxide, when the lipochrome pigments, together with a considerable amount of chlorophyll and sterols, passed into solution. The extraction of the dehydrated material was then completed by warming for a few hours under reflux with a mixture of equal parts of acetone and ether. The combined extracts were diluted with water and exhaustively extracted with ether. The ethereal extract was dried over sodium sulphate and solvent removed under reduced pressure in an atmosphere of nitrogen. As the pigments are, when impure, very sensitive both to heat ($>50°$) and to atmospheric oxygen, these precautions are very necessary.

Resolution of the lipoid materials. The removal of chlorophyll from the algal lipoids was accomplished by saponification with alcoholic potash (cf. Kuhn and Brockmann 1932*a*), except in those cases where fucoxanthin was present, as this pigment undergoes rapid alteration in the presence of alkali. The presence of fucoxanthin was ascertained by treating a small portion of the ethereal extract with 25% hydrochloric acid with which the pigment develops an intense blue colour in the lower acid layer (Willstätter and Page 1914).

(*a*) *Fucoxanthin absent.* The total residue was saponified by warming at 40° for several hours with 10% methyl alcoholic potash (10 c.c. to 100 mg. total lipoid) until a test portion diluted with water and extracted with ether showed a pure yellow colour in the ethereal layer, indicating complete saponification of the chlorophyll. The whole was then diluted with water, extracted with ether, and the ethereal solution thoroughly washed and dried. After removal of the solvent, the residue was dissolved in a mixture of equal parts of light petroleum (b.p. 40–60°) and methyl alcohol, and sufficient water added to dilute the latter to 90%. Under these conditions the hydrocarbon pigments, together with hentriacontane and the greater part of the sterols and terpenoid material, partition into the light petroleum, while the xanthophylls (polyene pigments containing hydroxyl groups) are retained in the methyl alcoholic phase.

(*b*) *Fucoxanthin present.* The total residue in this case was directly subjected to the phase separation, whereby fucoxanthin and other xanthophylls, together with small quantities of chlorophyll, pass into the aqueous methyl alcohol. The light petroleum solution was then evaporated and the residue, containing the sterols and epiphasic pigments, saponified as described above.

Unsaponifiable matter from light petroleum phase. The residue, usually a brown waxy material and varying in amount from 10 to 500 mg., was extracted with successive quantities (*ca.* 10 c.c.) of warm methyl alcohol until the extract showed signs of considerable coloration with carotenoid pigment. The clear hot solutions were bulked and cooled in the ice-chest when hentriacontane gradually separated and was removed by filtration (cf. Heilbron, Phipers and Wright 1934). In certain of the Chlorophyceae, e.g. *Pleurococcus Naegelii* and *Vaucheria hamata*, the amount of hentriacontane was large, and its presence rendered the isolation of the sterols exceedingly difficult. The filtrate was concentrated to about half its volume and cooled to − 10° when the sterols separated and were purified by repeated crystallization from methyl alcohol. In many cases, owing to paucity of material, the identification of the individual sterol had to depend solely on its melting-point. Where possible, however, its characterization was confirmed by the preparation both of its acetate and corresponding acetyl bromide (Windaus and Hauth 1906).

When fucosterol was present it was readily obtained as needles from methyl alcohol, giving a melting-point of between 121 and 124° and an acetate m.p. 119° which separated as plates from methyl alcohol, in good agreement with the findings of Heilbron, Phipers and Wright (1934).

The precise identification of sitosterol presented more difficulty. It is well known that the ordinary sitosterol of the phanerogams is not homogeneous and comprises a mixture of closely related compounds differing somewhat in melting-point and in optical rotation (Anderson 1926; Bonstedt 1928; Wallis and Fernholz 1936; Simpson and Williams 1937; Heilbron, Parry and Phipers 1935). It was similarly observed by Heilbron, Parry and Phipers (1935) that the sitosterol isolated from algae was not homogeneous and this we have now confirmed, the sitosterol obtained varying in melting point between 127–138°, whilst the steryl acetate had generally a melting point of 134–136°.

Epiphasic pigments. The residual gum left after the extraction of the sterols with methyl alcohol was dissolved in light petroleum and the solution repeatedly washed with water to remove all traces of methyl alcohol. After drying over sodium sulphate it was passed through a column of alumina (Merck, standardized according to Brockmann) whereby the pigment (or pigments) was retained on the upper part of the column, whilst hentriacontane and terpenes passed through and were collected in the filtrate; the chromatogram was then developed by washing with light petroleum (or a mixture of benzene and light petroleum). In all cases, with the exception of the three members of the Myxophyceae and of the green alga *Zygnema pectinatum* which will be dealt with in detail below, the completed chromatogram showed a single orange zone. This was eluted with benzene-methyl alcohol, and after washing several times with water and drying, the eluate was evaporated. Where possible the pigment was crystallized from benzene-methyl alcohol and characterized as carotene by its melting-point, crystalline form and absorption maxima. In cases where the quantity of pigment was insufficient to permit of its isolation the spectroscopic evidence had to be treated with caution, since Gillam and El Ridi (1936) have shown that the carotenes readily undergo isomerization. According to these authors this phenomenon is due to the action of the absorbent, but Zechmeister and Tuzson (1938) have shown that the conversion of *β*-carotene into its isomeride, *pseudo*-*α*-carotene, is spontaneous. A typical example of this phenomenon has been found in the red alga *Ahnfeltia plicata*. The carotene obtained from this was first adsorbed upon a column of alumina and subsequently resolved into its *β*- and *α*-isomers by the very convenient method of Strain (1934), which consists of the adsorption of the pigment upon magnesium oxide. The *β*-carotene obtained in this way simulated *α*-carotene in its spectroscopic properties, exhibiting absorption maxima at 508 and 477 mμ in carbon disulphide, values in absolute agreement with its isomeride *pseudo*-*α*-carotene. These values were not affected by readsorption of the pigment, but by crystallization from benzene-methyl alcohol the reversible isomerization was effected and plates of *β*-carotene were obtained showing the normal absorption maxima at 514 and 484 mμ.* In most cases the total carotene was isolated directly by elution, evaporation and crystallization. The composite nature of the carotene so obtained was shown by the low melting-point, usually about 172° (cf. Willstätter and Mieg 1907) in contradistinction to that of pure *β*-carotene, m.p. 184°.

Treatment of the methyl alcoholic phase—hypophasic pigments. The methyl alcoholic phase was largely diluted with water and extracted with ether. After removal of solvent from the dried solution, the oily residue consisting mainly of carotenoid pigments (only a very small quantity of sterol is present in this phase) was dissolved in benzene-light petroleum, and the solution passed through a column of alumina. The chromatogram was then developed by washing with further quantities of solvent, when, if more than one pigment were present, resolution into a series of coloured zones was effected. These were eluted separately and the individual pigments purified by readsorption upon alumina, and when the quantity of material permitted, isolated in the crystalline state. The most commonly occurring pigments were found to be "xanthophyll" and fucoxanthin.

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* The spectroscopic data referred to in this investigation have been obtained by use of a Hilger-Nutting spectrophotometer and except where otherwise stated the absorption maxima recorded refer to the use of carbon disulphide as solvent. It will be noticed that the values given for the first absorption maximum show a displacement towards the shorter waves of about 7 mμ from the values recorded upon grating instruments (cf. Hausser, Kuhn, Smakula and Kreuchen 1935). Thus the values obtained for the absorption maxima of *β*-carotene are 514 and 484 mμ and for *α*-carotene 507 and 477 mμ; Strain (1934) gives *β*-carotene 511 and 485 mμ and *α*-carotene 507 and 476 mμ; whilst Kuhn and Lederer (1931) give *β*-carotene 521 and 485·5 mμ and *α*-carotene 511, 478 mμ.

"*Xanthophyll.*" This pigment was identified by the yellow zone which it forms upon alumina, and by its optical maxima at 506, 473 and 441 mμ (Kuhn, Winterstein and Kaufmann (1930) record values of 505, 473 and 441 mμ). It consists principally of the well-defined carotenoid lutein, but small amounts of closely related pigments are present (compare Kuhn, Winterstein and Lederer 1931). It is to the presence of these that the shorter-waved absorption and low melting-point, *ca.* 173° (Willstätter and Stoll 1913), recorded are to be traced (pure lutein has maxima at 508 and 476 mμ and m.p. 193°). When the quantity of material permitted, the pigment was isolated by crystallization from methyl alcohol, when the typical swallow-tail crystals of "xanthophyll" were obtained (Willstätter and Stoll 1913). Where the solid pigment could not be obtained the identification of the carotenoid depends upon the absorption data. In many cases maxima in the neighbourhood of 500 and 472 mμ were noted; where, however, sufficiency of material enabled the pigment to be recrystallized the absorption maxima of the solid obtained had the usual values. The shift towards the shorter wave-lengths may be due, as in the case of β-carotene mentioned above, to isomerisation, for Zechmeister and Tuzson (1939) have shown that lutein (xanthophyll) readily isomerises to neolutein characterized by absorption maxima at 501 and 468 mμ. It has frequently been observed in the course of this investigation that xanthophyll gives on alumina two or more zones instead of one. It is possible that this phenomenon, doubtless due to the formation of isomerides, is the explanation of some of the results obtained by Kylin (1927), e.g. the presence of the so-called phylloxanthin or phyllorhodin which he considered to accompany xanthophyll in all green leaves. In an earlier paper (Heilbron, Parry and Phipers 1935) it was stated that the hypophasic carotenoid fraction of *Oedogonium*, *Cladophora Sauteri*, *Nitella opaca* and *Rhodymenia palmata* contained taraxanthin, $C_{40}H_{56}O_4$. The evidence for this was the appearance of a pigment with absorption maxima at 502, 472 and 442 mμ (absorption maxima of pure taraxanthin 501, 469 and 441 mμ (Kuhn and Lederer 1932)). It is to be noted, however, that the pigment was never isolated in the analytically pure state, and in view of the spectroscopic discrepancies mentioned above, as also from the fact that in none of the algae examined in the present paper has any evidence of the presence of taraxanthin been found, it is likely that the pigment is actually neolutein A or B.

Fucoxanthin. As mentioned above when fucoxanthin is present the use of saponification to remove chlorophyll is precluded, and in these cases the pigment was separated chromatographically, as described by Heilbron and Phipers (1935). The application of this method yielded fucoxanthin as a microcrystalline brick-red powder, m.p. *ca.* 150°, and showing absorption maxima at 508 and 474 mμ. Certain cases were observed where, instead of a single crimson band of fucoxanthin, two zones were obtained in the developed chromatogram. The upper, containing relatively little pigment, exhibited a somewhat blurred fucoxanthin spectrum and gave in ethereal solution a red-violet colour with 25% hydrochloric acid, gradually changing to blue; unlike the lower zone, no crystalline pigment could be obtained from it. The pigment present in this upper zone is in all probability the fucoxanthin-β described by Kylin (1927), and, as shown by Heilbron and Phipers (1935), is probably partially oxidized fucoxanthin.

CHLOROPHYCEAE

Enteromorpha compressa Grev. An ethereal solution of the lipoids gave no blue colour with 25% hydrochloric acid showing the absence of fucoxanthin, and the chlorophyll was therefore removed as described under (*a*) above. Extraction of the epiphasic portion of the unsaponifiable fraction with methyl alcohol yielded a minute quantity of colourless waxy solid, almost certainly hentriacontane (m.p. *ca.* 55°). Concentration of the methyl alcoholic solution gave sitosterol as plates (from methyl alcohol), m.p. 136–137°, acetate m.p. 134–135°.

The epiphasic pigments were adsorbed from light petroleum on to a column of magnesium oxide followed by development of the chromatogram with the same solvent, when two pigmented zones were obtained, the upper of which (orange coloured), after elution with benzene-methyl alcohol and repeated crystallization from the same solvent mixture, yielded β-carotene, m.p. 176° (uncorr.) (absorption maxima at 514 and 484 mμ). The small lower yellow zone contained insufficient pigment to permit of its isolation in the crystalline state, but its position in the chromatogram and the absorption maxima at 509 and 478 mμ show it probably to be α-carotene.

The hypophasic carotenoids were adsorbed on alumina from benzene solution, and after development with the same solvent three distinct zones were obtained, each having the same absorption maxima (504 and 475 mμ). Owing to paucity of material no solid pigment could be obtained, but it is almost certainly a lutein mixture.

Ulva lactuca Linn. was found to contain the same lipoids as the above.

Trentepohlia aurea Mart. The alga was extracted in the usual manner, and after transferring to ether and removal of solvent the residue was partitioned between 90% methyl alcohol and light petroleum when the major portion of the pigments passed into the upper phase. The light petroleum solution was saponified by refluxing with an equal volume of 5% methyl alcoholic sodium hydroxide for 2 hr.; under these conditions the red sodium salt of "haematochrome" if present would separate out at the interface (Zopf 1895), but none was observed. The mixture was diluted with water and the dried light petroleum solution examined as described in the general section. Small quantities of hentriacontane were obtained but no sterol could be detected. The pigmented residue was adsorbed on alumina from light petroleum when a single orange zone was formed from which by elution with benzene-methyl alcohol and crystallization from the same solvent, β-carotene was obtained as glistening plates, m.p. 177–178° (uncorr.), optical maxima at 513 and 483 mμ. The β-carotene content of *Trentepohlia aurea* was estimated spectroscopically and found to be 0·2% of the dry weight of the alga. The methyl-alcoholic phase was saponified in the usual manner and the unsaponifiable fraction examined chromatographically on alumina. A single orange zone was formed, the pigment of which was spectrographically identified as lutein (maxima at 506 and 473 mμ). We find that as observed by Heilbron, Lythgoe and Phipers (1935) the amount of xanthophyll present in *Trentepohlia aurea* is abnormally small.

Pleurococcus Naegelii Chod. The extract of this alga was resolved according to method (*a*). The epiphasic portion was unusually rich in hentriacontane which separated as a waxy solid from methyl alcohol, m.p. 60–62°. Only a very minute amount of sterol was obtained which crystallized from methyl alcohol in plates, m.p. *ca.* 127°; further purification was precluded by lack of material, but the melting-point indicates the presence of sitosterol. The carotenoid pigments were resolved in the usual way; from the epiphase β-carotene was separated and identified by its behaviour on adsorption on alumina and by its optical maxima (512 and 482 mμ). The hypophase yielded lutein with absorption maxima at 507 and 475 mμ.

Vaucheria hamata Lyngb. Two samples collected at different periods were examined independently. In ethereal solution the extracts of this alga gave a blue colour with 25 % hydrochloric acid; it was therefore treated as described under (*b*) for algae containing fucoxanthin. The unsaponifiable fraction of the light petroleum phase gave on resolution in the usual manner (*a*) carotene (maxima at 514 and 482 mμ), (*b*) hentriacontane, m.p. 60°, and (*c*) sitosterol, m.p. 128–130°. The hypophasic lipoids were dissolved in benzene and the solution passed through a column of alumina. After washing with benzene the chromatogram showed four zones: The main red zone (II) was eluted with benzene-methyl alcohol and the eluate evaporated to dryness and purified by readsorption on alumina from benzene. The pigment which formed a single homogeneous zone was isolated by elution with benzene-methyl alcohol and evaporation to dryness; it formed an oily residue which could not be induced to crystallize. This pigment gave a blue colour with 25 % hydrochloric acid and showed absorption maxima at 500 and 470 mμ indicating the presence of violaxanthin, $C_{40}H_{56}O_4$, a pigment shown by Kuhn and Winterstein (1931) to have absorption maxima at 501 and 469 mμ and to give a deep blue colour reaction with hydrochloric acid. In view of the unexpected occurrence of this pigment and in order to ensure that it was not in fact fucoxanthin, it was refluxed for 1 hr. with excess alcoholic potash, under which conditions fucoxanthin would be converted into *iso*fucoxanthin with absorption maxima at 474 and 448 mμ (Heilbron and Phipers 1935). The pigment was isolated by means of ether and the ethereal solution treated with N/10 acetic acid which develops a blue colour with *iso*fucoxanthin. As, however, the pigment gave no colour reaction and showed the same optical maxima as before treatment with alkali, the possibility of its being fucoxanthin is

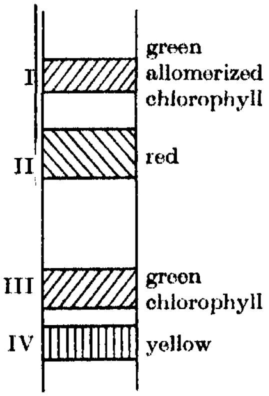

FIGURE 1

excluded. The pigment of the lowest zone (IV) was identified spectrographically as lutein.

Zygnema pectinatum (Vauch) Ag. As an ethereal extract of the lipoids gave a blue colour with 25 % hydrochloric acid, the alga was resolved as in the case of *Vaucheria*. Two colourless constituents were isolated from the light petroleum phase: (*a*) Hentriacontane (pearly plates from methyl alcohol, m.p. 60°), and (*b*) sitosterol which after two recrystallizations from methyl alcohol separated as plates, m.p. 134–136°, acetate m.p. 128°. The pigments present in this phase were resolved by chromatographing on a column of alumina; the complete chromatogram showed two zones, the lower of which was orange in colour and contained the bulk of the pigment. Elution with benzene-light petroleum and crystallization of the residue from the same solvent furnished β-carotene m.p. 174°, maxima 511 and 481 mμ in light petroleum. The more strongly retained upper zone which was bright red in colour was complex in nature, and insufficient was present for further investigation. The pigments in the alcoholic phase were adsorbed on alumina from benzene solution, the chromatogram being developed with the same solvent. It exhibited the zones shown in the annexed diagram. The pigment of zone I was obtained by elution with benzene-methyl alcohol and evaporation of the eluate; it gave in ethereal solution a reddish violet coloration with 25 % hydrochloric acid and exhibited ill-defined absorption maxima at 508 and 474 mμ. It is therefore probably partially oxidized fucoxanthin (cf. Heilbron and Phipers 1935). The bulk of the pigment which was present in zone II was purified by readsorption on alumina and crystallized from ether-light petroleum

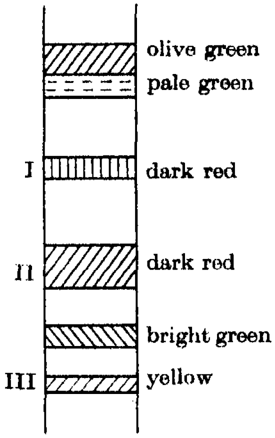

FIGURE 2

yielding microscopic red crystals, m.p. 147–150°, showing absorption maxima at 508 and 475 mμ, and giving a blue colour with 25 % hydrochloric acid in ethereal solution. Further evidence that this pigment was in fact fucoxanthin was obtained by refluxing it for 2 hr. with alcoholic potassium hydroxide; the resulting pigment was isolated by dilution with water and ether extraction, and gave on treatment, even with dilute hydrochloric acid, the characteristic blue colour reaction of the *iso*-fucoxanthins (Heilbron and Phipers 1935). Zone III was eluted with benzene-methyl alcohol and the eluate evaporated to dryness leaving a very small residue which showed maxima at 503 and 472 mμ and gave no blue coloration with concentrated hydrochloric acid in ethereal solution; it is therefore probably a mixture of lutein and neolutein.

XANTHOPHYCEAE

Botrydium granulatum (L.) Grev. An ethereal solution of the combined lipoid extracts imparted a deep blue colour to 25 % hydrochloric acid, and consequently resolution according to method (*b*) was adopted. The epiphasic lipoids were saponified in the usual way and furnished hentriacontane, sitosterol (plates from methyl alcohol, m.p. 134–135°) and β-carotene (maxima at 514 and 485 mμ). The hypophasic pigments were adsorbed from benzene solution on to a column of alumina, and after development with the same solvent produced a complex chromatogram of many zones. Owing to the extremely small quantity of material, complete separation of the chlorophyll could not be effected, and therefore the pigment of the main zone (red-brown) was eluted and saponified. The unsaponifiable fraction gave a strong blue colour with 25 % hydrochloric acid, but no colour with dilute hydrochloric acid, thus establishing the absence of *iso*fucoxanthin. The pigment exhibited well-defined absorption maxima at 475 and 447 mμ, values in exact agreement with those recorded by Kuhn and Brockmann (1932*b*) for flavoxanthin $C_{40}H_{56}O_3$, the pigment of the buttercup. No other natural pigment has so far been recorded having absorption maxima in this region.

BACILLARIOPHYCEAE

Nitzschia closterium (Ehr.) W. Smith. The non-saponifiable fraction of extracts of this alga, kindly furnished to us by Dr J. A. Lovern of the Torry Research Station, Aberdeen, was subjected to the phase partition between light petroleum and 90 % methyl alcohol. The epiphase was evaporated to dryness and treated in the usual manner yielding hentriacontane and a crude sterol, m.p. 106–108°. This was acetylated, fractional crystallization of the acetate yielding fucosteryl acetate (m.p. and mixed m.p. with an authentic specimen 119°) and a minute quantity of an unidentified steryl acetate which separated from methyl alcohol in small plates, m.p. 149–151°. The pigment present in this phase was identified spectroscopically as β-carotene. The hypophasic pigments were adsorbed upon calcium carbonate from a benzene-light petroleum solution and resolved by washing into two distinct zones. The pigment of the upper zone, which could not be obtained crystalline, gave a deep blue colour with *dilute* hydrochloric acid. It exhibited absorption maxima at 477 and 449 mμ and is therefore *iso*fucoxanthin, the presence of which can only be attributed to the action of the alkali employed in the saponification upon fucoxanthin originally present in the diatoms. The lower zone furnished by crystallization from methyl alcohol a small quantity of lutein, m.p. 176–178°, showing absorption maxima at 508, 475 and 442 mμ.

CHRYSOPHYCEAE

The algal material was kindly obtained for us from chalk cliffs at Westgate-on-Sea by Professor F. E. Fritsch, F.R.S., who identified the incrustation as being a mixture of three members of this class, viz. *Apistonema Carteri* Anand., *Thallochrysis litoralis* Anand., and *Gloeochrysis maritima* Anand. As the presence of fucoxanthin was indicated the lipochromes were resolved without preliminary saponification (method (*b*)). The epiphasic material contained β-carotene, identified by its absorption maxima at 514 and 485 mμ. The hypophasic pigments were separated by adsorption upon a column of calcium carbonate from carbon disulphide solution and identified spectroscopically as fucoxanthin and lutein. By acetylation and bromination, the crude sterol (which melted over a wide range) yielded a less soluble bromide, identified as fucosteryl acetate tetrabromide, m.p. 133° (Heilbron, Phipers and Wright 1934) and a more soluble bromide, m.p. 191–193°, which has not been identified.

PHAEOPHYCEAE

Fucus ceranoides Linn. Two large hauls of material obtained from different localities were examined and both found to contain the same lipoid constituents. The ethereal solution of the extracts of this alga (and indeed of all the Phaeophyceae) gave the blue colour reaction for fucoxanthin on treatment with 25% hydrochloric acid, and accordingly the analysis of the lipoid constituents was carried out by method (*b*) described in the experimental section. The sterol was obtained by recrystallization from methyl alcohol as plates, m.p. 120–121°, and formed an acetate m.p. 118–119° indicating the presence of fucosterol. A paraffin, probably hentriacontane, was obtained as a waxy solid (m.p. *ca.* 55°) and β-carotene was isolated chromatographically and identified by its absorption maxima at 514 and 484 mμ. The chromatogram of the hypophasic pigments showed a single crimson zone from which fucoxanthin was obtained by elution with benzene-methyl alcohol, evaporation of the eluate to dryness and crystallization of the residue from ether-light petroleum. No trace of any xanthophyll could be detected.

Ascophyllum nodosum Le Jol., *Laminaria digitata* Lamour, *Chorda filum* Lamour, *Halidrys siliquosa* Lyngb. and *Cladostephus spongiosus* J. G. Agardh. These algae were all resolved as described above for *Fucus ceranoides* and found to contain exactly the same lipoids.

Sphacelaria cirrhosa Agardh., *Stypocaulon scoparium* Kütz and *Dictyota dichotoma* Lamour yielded the same epiphasic lipoids as the above Phaeophyceae, but a definite difference in the hypophasic pigments has been observed. The latter were resolved by chromatographic analysis upon a column of alumina from benzene solution when four distinct zones were formed. The top olive-green zone was allomerized chlorophyll, while the second zone (crimson) consisted of partially oxidized fucoxanthin. Zone III (crimson) contained the bulk of the pigment present in the hypophase; this was identified as fucoxanthin by its optical maxima (508 and 474 mμ), and colour with 25% hydrochloric acid. The chlorophyll zone (IV) was observed to have a pure yellow lower edge not observed in other members of the class. As further separation of this yellow pigment by adsorption methods was unsuccessful, the chlorophyll was removed by saponification and the pigment transferred to ether and worked up in the usual manner. Its colour and position in the chromatogram definitely point to its being xanthophyll, but unfortunately too little material was present for further identification.

Pilayella littoralis Kjellm., *Ectocarpus tomentosus* Lyngb. The epiphasic lipoids were hentriacontane (plates from methyl alcohol, m.p. 60°), fucosterol m.p. 124°, and carotene m.p. 172°. The hypophasic fractions of both these algae contained an unusually large quantity of fucoxanthin. The pigments were separated by adsorption from benzene solution upon a column of alumina when five distinct zones were formed on washing with the same solvent. The first three of these contained the same pigments as for *Sphacelaria cirrhosa* and *Stypocaulon scoparium*, viz. allomerized chlorophyll, partially oxidized fucoxanthin and fucoxanthin. The latter was obtained as needles from ether-light petroleum, m.p. 156–157°. (Found: C, 75·7; H, 8·9. Calc. for $C_{40}H_{56}O_6$, C, 75·9; H, 8·9 %.) Immediately below the chlorophyll zone and separated from it by a clear space, a strong yellow zone was observed which yielded its pigment on elution with benzene-methyl alcohol. Although insufficient was present for isolation of a crystalline specimen, the pigment was identified as lutein by its well-defined optical maxima at 508 and 476 mμ.

RHODOPHYCEAE

Chondrus crispus Lyngb., *Gigartina stellata* Batt., *Phyllophora membranifolia* J. G. Agardh., *Plocamium coccineum* Lyngb., *Polysiphonia fastigiata* Grev. and both haploid and diploid phases of *Ceramium rubrum* Agardh. As the lipoid extracts of these algae gave negative tests for fucoxanthin, the chlorophyll was removed by saponification in the usual manner. In each case the epiphase yielded a paraffin hydrocarbon together with sitosterol m.p. 132–134°, acetate m.p. 134°. The epiphasic carotenoid present was chromatographed upon a column packed with a mixture of equal parts of magnesium oxide (micron) and siliceous earth (hyflo-supercel), when a small yellow zone was formed immediately beneath a main orange band, indicating the presence of β-carotene (absorp. max. 514 and 484 mμ) together with traces of either α-carotene or *pseudo*-α-carotene. The hypophasic carotenoid was in each case purified by adsorption upon alumina when a single orange zone was formed, the pigment from which was spectroscopically identified as lutein (absorption maxima at 506 and 477 mμ).

Dilsea edulis Stackh. and *Polyides rotundus* Grev. Insufficient sterol was present in the small quantities of these algae which were available to permit of its characterization; the pigments present were those described above for *Chondrus crispus*, etc.

Lemanea (*Sacheria*) *mamillosa* Kütz yielded in the epiphase (*a*) β-carotene as plates from benzene-methyl alcohol, m.p. 172–174°, maxima in carbon disulphide at 514 and 485 mμ, (*b*) α-carotene or *pseudo*-α-carotene (not obtained crystalline) giving maxima at 506 and 477 mμ and (*c*) sitosterol m.p. 132–135°, acetate m.p. 134°. The hypophasic pigments were adsorbed from benzene solution on to a column of alumina, and after development with the same solvent the chromatogram showed two separate zones. The pigment of the upper zone, m.p. 170–173° (from benzene-light petroleum), showed the characteristic maxima for lutein at 506 and 477 mμ, while the lower zone (purple) was shown by readsorption to be of a complex nature and no definite pigment could be isolated.

Ahnfeltia plicata Fries. The epiphasic carotenoids were adsorbed from solution in light petroleum on a column of magnesium oxide (micron) and siliceous earth (hyflo-supercel) and after exhaustive washing with light petroleum showed two zones, an upper orange one and a lower and much smaller yellow zone. The pigment from the former was crystallized from light petroleum when plates of β-carotene were obtained m.p. 179° exhibiting maxima at 514 and 484 mμ. The lower zone yielded a pigment with absorption maxima at 500 and 472 mμ. This was crystallized from alcohol to give a minute quantity of solid which showed absorption maxima at 508 and 476 mμ (probably α-carotene). Repeated crystallization of the crude sterol from methyl alcohol yielded a sterol crystallizing from methyl alcohol in small needles, m.p. 147°; acetate, m.p. 120–121°; acetyl bromide, m.p. 120° (possibly ε-sitosterol; cf. Simpson and Williams 1937). The hypophasic carotenoid was identified as lutein, m.p. 188° (corr.), maxima at 509 and 474 mμ.

Corallina officinalis Linn. Two specimens of this alga were examined, the one from regions about low-water mark and the other from rock pools at about mid-tide level; both gave the same results on examination of the lipoid constituents. The analysis was carried out as described for *Chondrus crispus*; the carotenoids were identical with those of this alga, but were only present in sufficient quantity to allow of spectroscopic detection. The sterol was in both cases obtained as needles (from methyl alcohol), m.p. 147°; acetate m.p. 120–121°, no depression was obtained in a mixed melting-point with the sterol obtained from *Ahnfeltia plicata*.

Polysiphonia nigrescens Grev. (both haploid and diploid phases). The ethereal solution of the total lipoid extract gave a deep blue colour with 25% hydrochloric acid and was therefore resolved as described in the experimental section under (*b*). The unsaponifiable fraction of the light petroleum phase furnished (*a*) β-carotene (absorption maxima at 514 and 484 mμ), (*b*) hentriacontane (plates from methyl alcohol, m.p. 58°) and (*c*) fucosterol separating after repeated crystallization from methyl alcohol as needles, m.p. 122–124°, and showing no depression in melting-point on admixture with an authentic specimen. The hypophasic carotenoids were freed from chlorophyll by adsorption on alumina when they formed an upper (red) zone and a lower (yellow) zone. The pigment of the upper zone was readsorbed on calcium carbonate from carbon disulphide solution, and after washing with this solvent formed a single homogeneous zone the pigment of which gave in ethereal solution a strong blue coloration with 25 % hydrochloric acid. By crystallization from ether-light petroleum it was possible to obtain a minute amount of a brick-red powder, m.p. 140–145° with maxima at 510 and 474 mμ; it is therefore undoubtedly fucoxanthin. The pigment of the lower yellow zone could not be obtained crystalline, but was spectroscopically identified as lutein (absorption maxima 508 and 472 mμ).

Porphyra umbilicalis J. G. Agardh. As no blue colour was given with 25 % hydrochloric acid, the material was resolved by method (*a*). The epiphasic lipoids comprised β-carotene, hentriacontane, m.p. 60°, and fucosterol, m.p. 120–121°. The hypophasic carotenoids comprised xanthophyll (identified spectroscopically) and a trace of a second less strongly adsorbed pigment which could not be further purified.

MYXOPHYCEAE

Rivularia nitida J. G. Agardh. This alga is a terrestrial species from the mud-banks of the Ynyslas salt-marshes of the River Leri.

The epiphasic lipoids worked up by method (*a*) formed a black wax-like mass from which no sterol could be isolated by extraction with methyl alcohol, although low-melting waxes separated on cooling the solution. The residue was dissolved in light petroleum and, after washing and drying, the solution was run through a column of alumina (Merck). The chromatogram was developed with light petroleum-benzene when three zones were produced and at the same time a yellow pigment passed through the column. The main purple zone showed a single broad band with head at 488 mμ corresponding with that of myxoxanthin (Heilbron, Lythgoe and Phipers 1935). The filtrate was evaporated and the residue crystallized from benzene-methyl alcohol from which β-carotene separated, m.p. 172°, maxima at 514 and 483 mμ. The hypophasic lipoids of the first batch examined contained insufficient pigment for detection, but from a subsequent batch, leaf xanthophyll was isolated separating from benzene-light petroleum as an orange powder (m.p. *ca.* 170°) with optical maxima at 506 and 477 mμ.

Rivularia atra Roth. This strictly marine species contained the same lipoids as *Rivularia nitida*.

Oscillatoria rubescens D.C. The epiphasic pigments present in this alga were identical with those of *Rivularia nitida*; the hypophase contained, in addition to xanthophyll, a second pigment (myxoxanthophyll) (Heilbron and Lythgoe 1936). No trace of sterol could be found, despite the relatively large amount of alga examined.

The analytical results described above are summarized for convenience in table 2.

DISCUSSION

The precise significance of the lipochrome pigments of the algae is as yet unknown although Senn (1911) and Geitler (1923) have suggested that they may act as a food reserve. Kuhn and co-workers (1938) have made the significant observation that they may have important functions to perform in the regulation of the reproductive processes. These authors were able to demonstrate that the copulation of the male and female gametes of the green alga *Chlamydomonas eugametos* which takes place on illumination of the aqueous suspension of the alga is achieved by the intervention of extraordinarily minute quantities of three substances: Crocin, which causes mobility of the cilia; an "initial stage" V, the activity of which is given by *cis*-crocetin dimethyl ester, and an "end-stage" Ko, the activity of which is given by *trans*-crocetin dimethyl ester; these are formed successively on irradiation of the algal suspension. The substance causing activation of the female gametes consists of a mixture of 3 parts of *cis*-ester and 1 part of *trans*-ester, and that responsible for the activation of the male gametes of 1 part of *cis*-ester to 3 parts of *trans*-ester which accounts for the observed order of the changes, viz. mobilization of cilia, activation of female gametes, activation of male gametes. Apart from this it is certain, however, that these photosynthetic pigments play an essential part in the metabolic and other processes of the plant; so much is implicit in the present system of algal classification, which is based to a large extent upon the pigmentation of the chromatophores. Closely related plants will have a similar cell metabolism and may reasonably be expected to synthesize the same pigments, an expectation supported by the work of Kylin (1927) who considered that specific pigments existed for each algal class. The accuracy of this view is borne out clearly by the present work, particularly by the uniformity in pigmentation amongst members of the same class, irrespective of order and family.

The most marked homogeneity in this respect is shown by the Phaeophyceae, which without exception contain carotene and fucoxanthin, despite the great diversity in the life cycles of the individual species examined. The only divergencies in pigmentation in this class are shown by both the members of the Ectocarpales examined (*Pilayella littoralis* and

TABLE II

Genus and species	Order	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
Chlorophyceae													
Ulva lactuca	Ulotrichales	+	.	.	+	.	.	.	.	.	+	.	.
Enteromorpha compressa	,,	+	.	.	+	.	.	.	.	.	+	.	.
Cladophora Sauteri	Cladophorales	+	.	.	+	.	.	.	.	.	+	+	.
Trentepohlia aurea	Chaetophorales	+	.	.	+	.	.	.	.	.	+	.	.
Pleurococcus Naegelii	,,	+	.	.	+	.	.	.	.	.	+	.	.
Oedogonium	Oedogoniales	+	.	.	+	.	.	.	.	.	+	.	.
Zygnema pectinatum	Conjugales	+	.	.	+	+	.	.	.	.	+	.	.
Vaucheria hamata	Siphonales	+	.	.	+	.	+	.	.	.	+	.	.
Nitella opaca	Charales	+	.	.	+	.	.	.	.	.	+	+	.
Xanthophyceae													
Botrydium granulatum	Heterosiphonales	+	.	.	.	.	.	+	.	+	+	.	.
Bacillariophyceae													
Nitzschia closterium	Pennales	+	.	.	+	+	.	.	.	.	.	+	+
Chrysophyceae													
Apistonema Carteri	Chrysomonadales	+	.	.	+	+	.	.	.	.	.	+	+
Thallochrysis litoralis	,,												
Gloeochrysis maritima	,,												
Phaeophyceae													
Pilayella littoralis	Ectocarpales	+	.	.	+	+	.	.	.	.	.	+	.
Ectocarpus tomentosus	,,	+	.	.	+	+	.	.	.	.	.	+	.
Sphacelaria cirrhosa	Sphacelariales	+	.	.	?	+	.	.	.	.	.	+	.
Stypocaulon scoparium	,,	+	.	.	?	+	.	.	.	.	.	+	.
Cladostephus spongiosus	,,	+	.	.	.	+	.	.	.	.	.	+	.
Dictyota dichotoma	Dictyotales	+	.	.	?	+	.	.	.	.	.	+	.
Chorda filum	Laminariales	+	.	.	.	+	.	.	.	.	.	+	.
Laminaria digitata	,,	+	.	.	.	+	.	.	.	.	.	+	.

Fucus ceranoides	Fucales	+	.	.	.	+	.	.	.	.	.	+	.
Fucus vesiculosus	"	+	.	.	.	+	.	.	.	.	.	+	.
Ascophyllum nodosum	"	+	.	.	.	+	.	.	.	.	.	+	.
Halidrys siliquosa	"	+	.	.	.	+	.	.	.	.	.	+	.
		Rhodophyceae											
Porphyra umbilicalis	Bangiales	+	.	.	+	.	.	.	.	+	.	+	.
Lemanea mamillosa	Nemalionales	+	.	.	+	.	.	.	.	+	+	.	.
Gelidium corneum	Gelidiales	+	.	.	+	.	.	.	.	.	+	.	.
Dilsea edulis	Cryptonemiales	+	.	.	+	.	.	.	.	.	Too little material available for identification		
Polyides rotundus	"	+	.	.	+	.	.	.	.	.			
Corallina officinalis	"	+	.	.	+	.	.	.	.	.	.	.	+
Polysiphonia fastigiata	Ceramiales	+	.	.	+	.	.	.	.	.	+	.	.
Polysiphonia nigrescens	"	+	.	.	+	+	.	.	.	.	.	+	.
Ceramium rubrum	"	+	.	.	+	.	.	.	.	.	+	.	.
Chondrus crispus	Gigartinales	+	.	.	+	.	.	.	.	.	+	.	.
Gigartina stellata	"	+	.	.	+	.	.	.	.	.	+	.	.
Phyllophora membranifolia	"	+	.	.	+	.	.	.	.	.	+	.	.
Ahnfeltia plicata	"	+	.	.	+	.	.	.	.	.	.	.	+
Rhodymenia palmata	Rhodymeniales	+	.	.	+	.	.	.	.	.	.	+	.
Plocamium coccineum	"	+	.	.	+	.	.	.	.	.	+	.	.
		Myxophyceae											
Oscillatoria rubescens	Hormogonales	+	+	+	+	.	.	.	+	+	.	.	.
Rivularia atra	"	+	+	+	+	.	.	.	.	.	.	.	.
Rivularia nitida	"	+	+	+	+	.	.	.	.	.	.	.	.

Column (1) Carotene; (2) Myxoxanthin; (3) Other epiphasic pigments; (4) Xanthophyll; (5) Fucoxanthin; (6) Violaxanthin; (7) Flavoxanthin; (8) Myxoxanthophyll; (9) Other hypophasic pigments; (10) Sitosterol; (11) Fucosterol; (12) Other sterols.

Ectocarpus tomentosus) and two out of the three Sphacelariales examined (*Sphacelaria cirrhosa* and *Stypocaulon scoparium*). These contain, in addition to carotene and fucoxanthin, small quantities of "xanthophyll" not possessed by higher members of the class; with increasing evolutionary development, the production of xanthophyll among the Phaeophyceae ceases completely. In this respect these higher orders of the brown algae differ from all other classes of algae and also from the phanerogams. It is well known that in their soma development and life cycles the Laminariaceae and the Fucaceae have reached, under marine conditions, a development comparable in some ways with that of the higher plants, and it is of interest to note the chemical changes which have accompanied these other evolutionary changes. The homogeneity of the Phaeophyceae as a class is further emphasized by the circumstance that every member contains the characteristic sterol, fucosterol. The significant occurrence of abnormally large amounts of fucoxanthin in *Pilayella* and *Ectocarpus* is almost certainly attributable to the simple anatomical form of the thallus, the presence of photosynthetic material in all the cells being due to absence of the tissue differentiation necessarily present in the bulkier plants.

Among the Chlorophyceae the normal photosynthetic polyene pigments are carotene and lutein (xanthophyll); only two exceptions to this rule have been found. Carotene and xanthophyll are the typical carotenoid pigments synthesized by the higher plants, a fact which gives strong support to the view that the Chlorophyceae are descendants of the forms from which the higher plants originated; thus Fritsch (1927) maintains: "It will be generally agreed that we must seek the origin of terrestrial plants in organisms possessing the same plastid pigments and the same essential metabolism as they do. The only representatives of such forms among the Protophyta at the present day are afforded by the numerous Green Algae." In regard to both sterols and lipochrome pigments the results of our investigation show that the Chlorophyceae, far more than any other class, approximate to the higher plants. Both in *Pleurococcus*, a terrestrial alga of doubtful affinities, and in *Oedogonium*, belonging to an order which according to Fritsch (1935) shows "but few points of affinity with the other Chlorophyceae", the pigmentation is normal.

For the behaviour of *Zygnema pectinatum*, which alone among the Chlorophyceae that we have examined synthesizes fucoxanthin in addition to carotene and xanthophyll, there is no obvious explanation. No other abnormality in the life cycle or habitat of this alga is known which might throw light on the occurrence of a pigment which is normally confined to the Phaeophyceae. This superficial resemblance to the brown algae is destroyed by the occurrence in Zygnema of sitosterol in place of the fucosterol characteristic of the Phaeophyceae. On the other hand, the divergence of *Vaucheria hamata* from the other Chlorophyceae was not unexpected. Botanists have long been aware of certain abnormalities in this member of the Siphonales. It is one of the few Chlorophyceae which regularly store their food reserve as oil instead of starch; it has been reported "to contain more xanthophyll than is usual in this class" (Printz 1927), and these facts, together with the unusual lateral arrangement of cilia on the spermatozoid, led some algologists to consider that *Vaucheria* was to be classified with the Xanthophyceae rather than with the Chlorophyceae. The occurrence in *Vaucheria* of violaxanthin, a pigment giving with strong hydrochloric acid the blue coloration by which the Xanthophyceae as a class are distinguished, seems at first sight to lend support to this view. It has been found possible to examine only one member of the Xanthophyceae (*Botrydium granulatum*), but the results indicate that the pigment responsible here for the coloration with hydrochloric acid is flavoxanthin, and not, as in *Vaucheria*, violaxanthin. In so far as the behaviour of *Botrydium* is representative of the whole class, therefore, no support can be given to the suggestion of an affinity between *Vaucheria* and the Xanthophyceae.

Turning to the sterols, here again the Chlorophyceae approximate to the land plants since all members contain sitosterol, although in *Nitella* and *Cladophora* it is accompanied by fucosterol. In an earlier communication (Heilbron, Parry and Phipers 1935), *Oedogonium* was the only member of the Chlorophyceae examined which contained sitosterol alone, but from our wider survey it now becomes probable that the simultaneous formation of fucosterol and sitosterol is confined to the Chlorophyceae and even in this class is the exception rather than the rule.

For the Chrysophyceae we find the occurrence of carotene, xanthophyll and fucoxanthin, together with fucosterol and a new sterol. In this respect they resemble *Nitzschia closterium*, the only member of the Bacillariophyceae which we have been able to examine. The only difference is the presence in this alga of an unidentified sterol which is different however from that present in the Chrysophyceae. Chemically, therefore, the Bacillariophyceae and Chrysophyceae stand in close relation to the more primitive Phaeophyceae (Ectocarpales). We realize however that chemical processes as well as the developmental sequences may be paralleled in quite diverse classes without necessarily implying any affinity. Pascher (1921) has suggested a relationship between the Bacillariophyceae, the Chrysophyceae and the Xanthophyceae. Among the physiological features which warrant this suggestion are the presence of a large amount of carotenoid pigment, the similarities in food reserve formed by the cells and the frequent deposition of silica in the bipartite cell-wall. In so far as the members examined may be regarded as typical of the classes of algae to which they belong, it may be concluded that there is evidence for an affinity between the Chrysophyceae and the Bacillariophyceae. The Xanthophyceae, however, appear to contain carotene, xanthophyll, flavoxanthin and sitosterol and show closest affinity to the Chlorophyceae.

The pigmentation of the Rhodophyceae is largely due to the water-soluble chromoproteins, and despite the wide variations in colour, a marked homogeneity is shown by the class, carotene and xanthophyll being found in every member examined. Divergencies from the normal in this class are very slight. In the more primitive members, namely the Protoflcoridean *Porphyra umbilicalis* and a fresh-water member of the Nemalionales, *Lemanea mamillosa*, there were suggestions of the presence of some other polyene pigment, reminiscent of the exceptional occurrence of xanthophyll amongst the Phaeophyceae. No evidence of the epiphasic myxoxanthin which is characteristic of the Myxophyceae was obtained, a fact militating against acceptance of the theory of relationship between the Protoflorideae and the Myxophyceae (Ishikawa 1921). Such a relationship is considered to be shown by the existence of both phycocyanin and phycoerythrin in the chloroplasts and by the primitive nature of the mitotic divisions, but does not accord with the presence in the Bangiales of a definite chromoplast with its associated pyrenoid and with the sexuality obtaining in this order. The most surprising abnormality in the Rhodophyceae is shown by *Polysiphonia nigrescens*. Unlike other species of the genus, this dark purple coloured alga contains fucoxanthin and fucosterol, as well as carotene and lutein, thus simulating chemically a member of the Ectocarpales. *Polysiphonia nigrescens* is otherwise a perfectly normal diplobiontic red alga with a habitat and life cycle similar to other members of the Ceramiales. The closely related *Polysiphonia fastigiata* is quite regular in its pigmentation in spite of the fact that, as a parasite on *Ascophyllum nodosum* the possibility existed of it obtaining supplies of fucoxanthin from its host. The dark purple colour of *Cystoclonium purpureum* Batt. suggested that fucoxanthin might also be present in this alga, but such was not the case.

The normal sterol of the Rhodophyceae appears to be sitosterol, but exceptions to the rule are many. Both *Porphyra* and *Polysiphonia nigrescens* contain fucosterol while both *Corallina officinalis* and *Ahnfeltia plicata* synthesize a peculiar sterol which approximates in some of its constants with the so-called ϵ-sitosterol of Simpson and Williams (1937).

Amongst the Myxophyceae which we have examined, all show the presence of carotene, myxoxanthin and xanthophyll. *Oscillatoria* also possesses other hypophasic pigments not present in the Rivulariaceae, notably myxoxanthophyll. Perhaps the most remarkable feature of this class is the absence of sterol; it is possible that this might be correlated with the lack of sexuality obtaining in this class, but the apparent absence of any sterol from the green alga *Trentepohlia* renders difficult any generalization.

It is interesting to note that in both haploid (sexual) and diploid (tetrasporic) phases the same pigments and sterols are present; this was shown for both *Ceramium rubrum* and for *Polysiphonia fastigiata*.

No effect of habitat on lipoid constituents could be traced amongst the plants examined. The same lipoids are synthesized among the Chlorophyceae by the marine *Enteromorpha*, by the fresh-water *Oedogonium* and by the terrestrial *Pleurococcus*. Among the Phaeophyceae the brackish water *Fucus ceranoides* is identical in pigments and sterols with the marine representatives of the class. Among the Rhodophyceae the fresh-water *Lemanea* does not diverge appreciably from the other marine-members of the class; moreover, *Corallina officinalis* from two different habitats on the shore, from low-water mark and from rock pools above mid-tide levels, yielded identical results.

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SUMMARY

1. The specific lipochrome pigments and sterols of representative members from seven of the eleven classes of the Algae have been examined.

2. The Chlorophyceae resemble the higher plants closely in their synthesis of carotenoid pigments which are normally carotene and lutein. Exceptions are *Zygnema pectinatum* which produces in addition fucoxanthin and *Vaucheria hamata* which contains violaxanthin.

3. The uniformity of pigmentation implied in the classification of the Algae is borne out by the present work, this being most marked in the Phaeophyceae which without exception synthesize carotene and fucoxanthin. Fucosterol is the characteristic sterol of this class.

4. The Bacillariophyceae and the Chrysophyceae show a close relationship to the more primitive orders of the Phaeophyceae.

5. The Rhodophyceae all contain carotene and lutein and there is no evidence of a relationship with the Myxophyceae all of which contain the characteristic epiphasic pigment myxoxanthin.

6. *Polysiphonia nigrescens* is an exceptional red alga in synthesizing fucoxanthin in addition to carotene and lutein. It also contains fucosterol.

7. The absence of sterols in the Myxophyceae may be correlated with the lack of sexuality in this class.

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The subjective judgment of the elastic and plastic properties of soft bodies; the "differential thresholds" for viscosities and compression moduli

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1. Introduction

In measuring the softness of materials which show both elastic and plastic properties, it is customary either to assess the softness and elasticity by means of purely empirical tests, or, more frequently in industrial processes, to rely on the subjective judgment of some experienced person. Little is known of the capacity of individuals to measure such properties by tactual, kinaesthetic and visual observations, save that Katz (1937) has made a preliminary study of the capacity of bakers to judge the properties of flour dough, and concludes that their special skill is not in the main due to any abnormal capacity to judge flow and deformation ("rheological") properties. Problems of this kind are to be found in many industries, notably bread and biscuit making, cheese and butter manufacture and ceramics.

On the theoretical side, the data from such complex materials have been fitted into a double framework originally devised for true fluids and elastic solids respectively. Equations were used containing two terms, one an essentially Newtonian term, but involving a variable viscosity, and the other an essentially Hookian term suitably modified to allow for elastic hysteresis and after-effect.* There is much to be said for this treatment. The properties so evaluated are obtained in c.g.s. units, and are reproducible so long as fixed arbitrary conditions of stress and strain are maintained. Moreover, physicists are accustomed to thinking in terms of viscosities and elastic moduli, and more readily assimilate information presented in such familiar terms. On the other hand, it must be remembered that to divide the phenomena of deformation into viscous and elastic parts involves a division of the strains produced into recoverable and non-recoverable components; and, since some materials show exceedingly slow elastic after-effects, such a distinction is often quite arbitrary. Moreover, for very complex systems such as are met with in nature and dealt with in industry, the Newtonian-Hookian framework is decidedly artificial.

An alternative way of analysing the deformations of those complex soft bodies in which Poisson's ratio does not differ appreciably from 0·5 would be to regard these deformations, not as a sum of Newtonian or Hookian components, but as *intermediate entities*, the true fluid and elastic solid being limiting cases. Thus, for a true fluid, we have, after Newton:

$$S = \eta\sigma t^{-1}, \tag{1}$$

where S is shearing stress,
σ is shearing strain,
t is time of application of stress,
η is viscosity (or σt^{-1} is rate of shear);

whereas for an elastic solid $S = n\sigma$, (2)

where n is the shear modulus

Empirically, one might write for an intermediate material:

$$S = \psi\sigma t^{-k}. \tag{3}$$

In this equation ψ is a measure of what may be called "firmness", and has dimensions depending on the value of k. k, which will be zero for an elastic solid, and unity for a Newtonian fluid, is a measure of the "elasticity" of the material (using the word in the colloquial sense). For dimensional reasons, and for convenience in calculation, it is preferred to write this equation:

$$\log S = \log \psi + \log \sigma - k \log t. \tag{4}$$

This is an equation with two unknowns, and can be solved by means of data from determinations of deformations produced by a constant stress, for a minimum of two intervals of time.[*] An apparatus is in process of construction in which cylinders of suitable materials can be loaded in such a way that the stress per unit area on the cylinder is kept constant during the loading in spite of the change in cross-section of the cylinder. The strains are recorded during the time of the loading.

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* E.g. Schofield & Scott Blair 1933*b* for flour doughs.

It is not supposed that ψ will be a constant for all materials independent of stress or strain conditions. The viscosities and moduli of the older methods have never been constants in this sense for such bodies, but the treatment here proposed seems more closely related to the main purpose of the investigation, which is to study the nature and limitations of subjective judgments of rheological phenomena, by comparing the results of these judgments with objective physical measurements.

Any alleged exceptional skill on the part of an expert to judge rheological properties of materials and to apply such judgments to practical ends, must be ascribed to a superior capacity in one or more of the following ways:

(1) Ability to distinguish small differences in rheological properties, of which viscosity and compression- or shear-modulus are typical.

(2) Capacity to recognize[†] rheological conditions previously associated with certain desirable or undesirable situations.

(3) Technical knowledge enabling an effective utilization of information derived from (1) and (2).

Before pursuing the investigation for highly complex systems, it seemed desirable to explore the first possibility by determining the capacity of individuals having different types of education and experience to distinguish small differences, in systems in which "firmness" depends on one physical factor, i.e. (*a*) viscosity of true fluids of high viscosity, and (*b*) compression modulus of elastic solids. These experiments form the substance of the present paper.

2. THE "DIFFERENTIAL THRESHOLD" FOR VISCOSITY

Some difficulty was experienced in procuring a suitable material for this work, and we are indebted to a number of firms for supplying samples of bitumen, pitch, etc. for trial. Finally, through the courtesy of the Limmer and Trinidad Lake Asphalt Co., Ltd., we were able to obtain a sample of Californian bitumen, which, when diluted with small quantities of Golden Flux Oil, gave a series of samples, almost Newtonian fluids, differing in viscosity over a suitable range, and having viscosities of the order of 10^6 poises at room temperature.

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[*] A superficially similar empirical equation has been used by Williams working on cylinders of rubber, but this author uses height of cylinder instead of strain and in this way his treatment lacks the formal connexion with the Newtonian and Hookian laws (Williams 1924). A number of other authors have used equations similar to that of Williams.

[†] "Recognize" is here preferred to "remember", *vide* Bartlett (1932). See also Section 4.

The determination of absolute viscosity, and the experiments to check that samples were adequately Newtonian, were carried out by the method of rheograms slightly modified (Schofield and Scott Blair 1933*a*). In brief, a cylinder of bitumen, of about 1 cm. diameter and of a convenient length, was laid on a short piece of wood, and stamped with a mm. scale by means of a threaded brass rod. The cylinder was then hung freely in a vertical position in a test tube in a thermostat. After a convenient time, it was carefully laid down on the wood again and a piece of paper, lightly damped with chloroform, was gently pressed on to the now distorted scale. This was repeated with cylinders of different lengths. The analysis of the "transfers" or "rheograms" so obtained was carried out as described in the earlier paper. A flow curve for one of the bitumen samples is shown in figure 1. It includes data from two experiments with different total strains. This is the high viscosity sample from which the others were prepared by dilution.

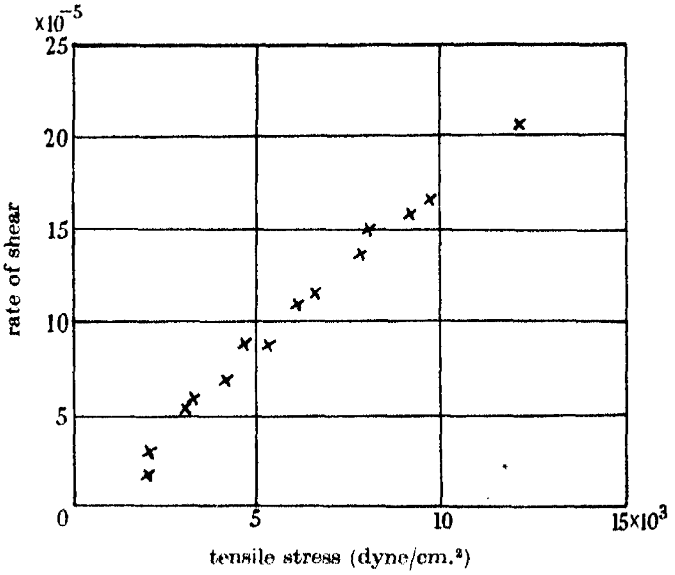

Figure 1

None of the classical psychophysical methods* was applicable to this experiment without considerable modification. The intensity of the stimulus cannot be progressively increased during the test, thus precluding the method of average error. The constant method, the method of limits and the method of serial groups would require more material than was available, or more time for experimentation than our subjects could give, nor would it have been practicable to use subjects for longer experiments on account of the "boredom" factor. Moreover, these materials change their physical properties rather rapidly with age. This precludes the possibility of doing any very large series of experiments. The same standard cannot be used repeatedly on account of the high viscosity-temperature coefficient. Since it was of interest to compare "thresholds" obtained for viscosities, directly with those for compression moduli (experiments described in Section 3), it was clearly important to use experimental procedures which could be applied to both viscous and elastic materials. Although by careful dilution with oil it was possible to prepare a series of bitumen samples differing in viscosity by equal amounts (within very narrow limits), evenly spaced samples could not be prepared for the compression modulus experiments. Since the distribution of samples was therefore not regular, the use of a method involving the selection of a single arbitrary standard was avoided. In view of all these circumstances, the experiment of which a preliminary description has been given (Scott Blair and Coppen 1939) was conducted as follows:

Thirteen subjects were chosen, having widely differing experience and training. For convenience, these will be designated as follows:

m = male;
f = female;
ϵ = university graduate, not science;
σ = scientific graduate;
τ = skilled in technological testing work (cheese manufacture);
ρ = routine analytical chemist.

The subjects ranged in age from 16 to about 45 years. In a few cases, where the ages are of interest, they are added in parentheses to the designations.

Samples of bitumen were prepared, differing in viscosity by 0, 7·5, 15, 22·5 and 30 %, and were presented to the subject in pairs, in random order,* one sample being placed in each hand. Each test consisted normally of 5 pairs for each interval. In a few cases, however, subjects confused the samples and one or two pairs were missed, since the samples in question could not be re-identified until after the test was completed. These cases are clearly marked in table 1. The samples were very approximately spherical in shape, and weighed about 18 g. The subjects were instructed to feel the samples by handling in any way they liked and allowed to use sight if they cared to do so, but told to keep the samples under water in a basin.† The water was changed from time to time to keep the temperature approximately constant. The subjects were told to change hands twice, notifying the experimenter at each change, and asked finally to report which, if either, sample was the softer. Though none of the subjects was trained in introspection, they were asked to give any information as to the methods used in forming a judgment. Subjects were told that a small proportion of the pairs would be indistinguishable ("equal"). In order to encourage cautious subjects, all subjects were asked to express an opinion if at all possible, even if very doubtful. For purposes of computation, all judgments were counted, whether expressed as "doubtful" or not. It is appreciated that these conditions are not ideal for psychophysical experimentation in general, but they appear to be the most satisfactory for this problem. Handling the bitumen out of water is very unsatisfactory owing to the high viscosity-temperature coefficient, and on account of stickiness. That the use of water did not seriously handicap judgments was shown in the second experiment, and will be discussed later. Subjects showed rather widely differing natural times for the test. The result of forcing a slow subject to work fast, or a fast subject to work slowly, was to raise the "threshold". There was, therefore, no restriction placed on subjects with regard to time. Several of the subjects were not able to spare the time to do the test very frequently, but the results from those who had done so showed little or no "learning" effect after the first or perhaps the second

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* These are enumerated in a number of text-books on psychology, e.g. Myers (1931).

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* Except that the same sample was never given twice within three tests because of possible temperature changes produced by handling. Between tests the samples were kept in water at constant temperature.

† This procedure implies a good deal of differential pressure over the skin areas affected, and this differential pressure is a variable and uncontrolled quantity. It is reasonable to hold that such differential reactions among a large mass of sensory receptors seriously affect threshold reactions. However, apart from the question of the effects of certain inevitable differences between the techniques used for viscous and elastic materials (*vide* Section 3), it has not been practicable to attempt to control or measure these factors. To do so would involve an enormously prolonged series of experiments and would remove the whole investigation too far from the practical realm.

TABLE 1

Subject	No. of tests n	Average time min.	Average* no. of judgments given "doubtful"	Pairs differing in viscosity by (%)					Proportion for all intervals except 0
				0	7·5	15	22·5	30	
m	1	40?	6	(*a*) 0‡	(*a*) 0·40	0·80	0·80	1·00§	0·74
					(*b*) 0	0	0	0	0
					(*c*) 0·60	0·20	0·20	0	0·26
m†	1	98	0	(*a*) 0	(*a*) 0·80	0·60	0·40	0·80	0·65
					(*b*) 0	0·20	0	0	0·05
					(*c*) 0·20	0·20	0·60	0·20	0·30
f (16)	2	44	0	(*a*) 0	(*a*) 0·90	0·90	0·90	0·90	0·90
					(*b*) 0	0	0	0	0
					(*c*) 0·10	0·10	0·10	0·10	0·10
$m\epsilon$	1	40?	5	(*a*) 0·20	(*a*) 0·20	0·20	0·80	0·80	0·50
					(*b*) 0·60	0·80	0·20	0·20	0·45
					(*c*) 0·20	0	0	0	0·05
$f\epsilon$	6	41	6·3	(*a*) 0·36‡	(*a*) 0·45§	0·61‡	0·59§	0·62§	0·58
					(*b*) 0·24	0·28	0·31	0·24	0·27
					(*c*) 0·31	0·11	0·10	0·14	0·15
$m\epsilon\sigma$	1	40?	4	(*a*) 0·80	(*a*) 0·20	0·20	0·80	0·80	0·50
					(*b*) 0·80	0·80	0·20	0·20	0·50
					(*c*) 0	0	0	0	0
$f\epsilon\sigma$	1	70	7	(*a*) 0·20	(*a*) 0·20	1·00	0·80	0·60	0·65
					(*b*) 0·40	0	0	0·40	0·20
					(*c*) 0·40	0	0·20	0	0·15
$m\sigma\rho$	6	67	1·9	(*a*) 0·28§	(*a*) 0·57	0·63	0·72§	0·86‡	0·69
					(*b*) 0·20	0·17	0·21	0·14	0·18
					(*c*) 0·23	0·20	0·07	0	0·13
$f\sigma\rho$	5	37	0	(*a*) 0·13‡	(*a*) 0·48	0·75§	0·71§	0·88	0·70
					(*b*) 0·12	0	0·04	0	0·04
					(*c*) 0·40	0·25	0·25	0·12	0·26
$m\epsilon\sigma\tau$	2	49	1·5	(*a*) 0‡	(*a*) 0·30	0·70	0·80	0·67§	0·62
					(*b*) 0·40	0·20	0·10	0·11	0·20
					(*c*) 0·30	0·10	0·10	0·22	0·18
$f\epsilon\sigma\tau$	2	32	2·5	(*a*) 0‡	(*a*) 0·60	0·50	0·60	0·89§	0·64
					(*b*) 0·30	0·20	0·20	0	0·18
					(*c*) 0·10	0·30	0·20	0·11	0·18
m (experimenter)	1	25	7	(*a*) 0·20	(*a*) 0·60	0·40	0·80	1·00	0·70
					(*b*) 0·20	0·20	0·20	0	0·15
					(*c*) 0·20	0·40	0	0	0·15
f (experimenter)	1	85	12	(*a*) 0·40	(*a*) 0·60	0·40	1·00	0·80	0·70
					(*b*) 0·20	0·40	0	0·20	0·20
					(*c*) 0·20	0·20	0	0	0·10
Means for all tests (excluding experimenters)				(*a*) 0·22	(*a*) 0·50	0·63	0·71	0·80	0·66
					(*b*) 0·23	0·20	0·15	0·12	0·18
					(*c*) 0·27	0·17	0·14	0·08	0·16

Total number of judgments given, 726.

* Including "very doubtful". These are means per test, i.e. of 25 pairs.

† The only manual worker.

‡ Subject missed two comparisons.

§ Subject missed one comparison out of $5n$ which should have been given for this interval.

test, except in reducing the natural time.[*] After about six tests, subjects began to show signs of boredom, and no subject was asked to do the test more than seven times. The same subject never did the test more than once in any given day. In a few cases the hands began to be sore after five or six tests. The actual methods of handling differed considerably for different subjects, and the use of sight was also very variable. The results are given in table 1 as the proportions judged (*a*) correctly, (*b*) as equal, and (*c*) in reversed order.

In table 1 (*a*) are given the P figures and the χ^2 data from which they were derived. These χ^2 figures were calculated from the data from all intervals except 0 because of the limited number of data for individual intervals in the case of some of the subjects.[†]

TABLE 1 (*a*)

	Individual variations	τ	Sex	σ	$\left\{\begin{array}{l}\epsilon\text{[‡]} \\ \text{age}\end{array}\right.$	ρ
χ^2	10·3	0·05	0·11	3·21	7·37	5·56
P	0·3	0·8	0·7	0·05	<0·01	0·02

We therefore conclude that there is no significant difference between individual subjects in their capacity to distinguish differences in viscosity in our experiments with truly fluid bitumens. There is no significant difference between groups of subjects differing in technological experience or sex. Scientific training is a borderline case, and experience in routine testing shows a significant advantage. Age and general education appear disadvantageous.

Inspection of table 1 indicates that female subjects tend to give less equal judgments than males. There is no appreciable difference in the number of doubtful judgments. One subject, *f* (16) (data not included in χ^2 analysis), appeared in two tests to be rather above the general level, but when tested again four months later she failed to repeat her performance, although this failure may have been due largely to the undoubted changes in the physical properties of the materials. Neglecting the two experimenter subjects, a general approximate threshold for viscosity may be obtained by taking a sum of all the tests. In view of the big differences found between the thresholds for different physical properties, such an approximate figure is of definite value.

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[*] It is likely, however, that the subjects rapidly develop some kind of functional standard which they can subsequently use. This point will be more fully discussed in Section 4.

[†] In testing individual variations from the average for ten subjects, the formula $\frac{1}{a+a'}(an'-a'n)^2$ was used, in conjunction with a ten by twofold contingency table. To test the variations of any group from the mean of the remainder, the formula $\frac{(ad-bc)^2\,(a+b+c+d)}{(a+b)\,(c+d)\,(a+c)\,(b+d)}$ was used, in conjunction with a two by twofold contingency table (*v.* Fisher 1936).

[‡] Since the majority of our educated subjects was also above the average age it is impossible to separate the effects of ϵ and age.

The table of summed data shows that the proportion of judgments given correctly exceeds 50 % for differences in viscosity of about 10 %, and reaches the 80 % level for differences of 30 %.

There is no evidence that any one method of handling the samples was particularly advantageous, nor did the use of sight or its voluntary elimination make any great difference to results. The best results were obtained by allowing each subject to use the method and rate of working natural to himself, in spite of the difficulties which this procedure is bound to introduce.

The experimenters themselves showed no superiority in carrying out the tests as subjects, although they alone understood the purpose and planning of the experiment.

3. THE DIFFERENTIAL THRESHOLD FOR COMPRESSION MODULUS

A number of cylinders of rubber 2·5 cm. in height and 2 cm. in diameter was supplied by the Research Association of British Rubber Manufacturers. As a result of a preliminary experiment, a set of ten of these was selected. Three pairs of these had been planned to be duplicates, but it was not found possible to prepare duplicate cylinders in which the compression moduli were insignificantly different. This was not disadvantageous, since whereas two of the supposedly equal pairs differed by a definitely subliminal amount and the third pair corresponded closely to the "threshold", several other pairs also differed by subliminal amounts.

From a preliminary experiment, it was clear that the "threshold" for compression modulus was likely to be exceedingly small, and a special apparatus was therefore constructed to measure the moduli with some accuracy. Comparative rather than absolute accuracy was required, so that a few somewhat arbitrary factors were introduced. A metal rod was soldered at one end to a rectangular plate, and to a gramophone needle at the other, indicating on a scale. A slot was filed in the plate which rested on a hard-steel knife-edge so as to form a beam 140·5 cm. in length from pointer to fulcrum. 5 cm. on the other side of the fulcrum were drilled two small holes in the metal plate through which wire cables passed, suspending a metal disk to which weights could be added. The rubber cylinder to be tested was placed on a firm stand midway between the cables, so that it was loaded directly from above its centre. Since there was a slight angular tilt in the compression due to the shortness of the loaded side of the beam, the weight of the long side was slightly over-compensated, the whole cylinder being very slightly under-load before the start of the experiment (see figure 2). In the experiment the cylinders were loaded with a weight of 20 lb. Rubber is not a perfectly elastic solid, and for this reason the times of loading and recovery, as well as the method of damping of the beam (which showed a good deal of "whip"), were very carefully standardized.

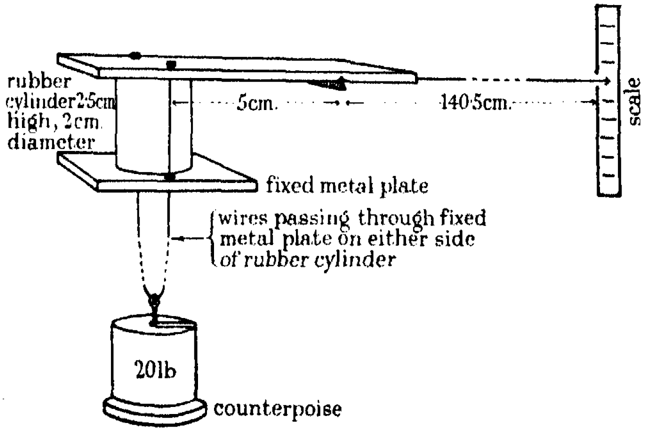
rubber cylinder 2·5cm high, 2cm diameter
5cm.
140·5cm.
scale
fixed metal plate
wires passing through fixed metal plate on either side of rubber cylinder
20lb
counterpoise

Figure 2

In preliminary tests some subjects began to recognize the cylinders from markings on the surface. All the cylinders were therefore uniformly dyed with gentian violet solution, identification being made possible for the experimenter by means of very small dots and dashes made in the side of the cylinder with a pin.

The moduli for all the cylinders used in the test were measured, and the ratios for each pair to be used were calculated. Replicate readings for a given cylinder agreed to within between ¼ and ½ %. The moduli were of the order of $1 \cdot 5 \times 10^7$ dynes/cm.^{2} After several preliminary experiments, ten subjects were chosen, of whom seven had been used in the viscosity experiment, three being new subjects. A main experiment was first conducted in which subjects were allowed to handle the cylinders in any way they wished, without a water thermostat.

We are indebted to Professor F. C. Bartlett, F.R.S., for the suggestion that the possible effects of (A) judging by means of the sharp edge of the cylinder, and (B) working under water, should be investigated. This was done in a subsidiary experiment to be described later. Subject to there being no serious errors from these sources, it seemed best to make a comparison between the "thresholds" for viscosity and compression modulus, giving the subject the utmost freedom of choice of method. Introspections and observations of method were made as before.

Since the differences in modulus given could not be controlled, the results of this test cannot be classified in quite the same way as was used for table 1.

The pairs may be grouped as follows:

TABLE 2

Group	1	2	3	4	5	6	7
Modulus difference %	0–2	2–4	4–6	6–8	8–10	10–12	12–14
No. of pairs	3	4	4	0	2	1	1
Mean modulus difference %	1·47	2·74	5·56	—	9·59	10·59	12·62

In each test fifteen pairs were given twice each, in random order, making a total of thirty comparisons per test. In table 3 are given the results of the main experiment. In this case all equal judgments are formally listed as incorrect, since objective differences could be observed in all cases. The figures in table 3 are the proportions given (*a*) correctly, (*b*) as equal, and (*c*) in reversed order.

In the subsidiary experiment, the two best subjects were asked to repeat the test, (A) squeezing the sides of the cylinder only, between the fingers and thumb so that the edge could not be felt, and (B) ditto, working also under water. One subject complained of fatigue in these tests and both subjects were therefore encouraged to rest the fingers as often as was necessary for a few moments. About three rests were taken during each test by *mσp*, but *fσp* did not require rests. The results are given in table 4.

The χ^2 tests were made as in the viscosity experiment, and the results are given in table 3 (*a*).

From tables 3, 3 (*a*) and 4 we conclude:

That, unlike the viscosity experiment, there are significant differences between individual subjects in their capacity to judge difference in compression modulus in our experiments with almost truly elastic rubber cylinders.

There is no significant difference between groups of subjects of different sex.

TABLE 3

Subject	No. of tests n	Average time min.	Doubtful judgments: means per test	Group	1	2	3	4	5	6	7	Proportion for all groups
m (16)	1	50	0	(a)	0·17	0·25	1·00	—	1·00	1·00	1·00	0·74
				(b)	0·66	0·62	0	—	0	0	0	0·21
				(c)	0·17	0·13	0	—	0	0	0	0·05
f (15)	1	42	3	(a)	0	0·75	0·63	—	1·00	0·50	1·00	0·65
				(b)	0·67	0·25	0·25	—	0	0·50	0	0·28
				(c)	0·33	0	0·12	—	0	0	0	0·07
f (16)*	2	33	8·5	(a)	0·25	0·56	0·56	—	0·88	0·75	0·75	0·63
				(b)	0	0	0	—	0	0	0	0
				(c)	0·75	0·44	0·44	—	0·12	0·25	0·25	0·37
$f\epsilon$	6	18	2	(a)	0·50	0·46	0·61	—	0·54	0·75	0·83	0·62
				(b)	0·03	0·08	0·10	—	0·21	0	0	0·07
				(c)	0·47	0·46	0·29	—	0·25	0·25	0·17	0·31
$m\epsilon\sigma$	2	38	8·5	(a)	0·33	0·13	0·44	—	0·63	1·00	1·00	0·59
				(b)	0·08	0·50	0·50	—	0·37	0	0	0·24
				(c)	0·59	0·37	0·06	—	0	0	0	0·17
$f\epsilon\sigma$*	2	50	3	(a)	0·50	0·56	0·82	—	1·00	1·00	1·00	0·82
				(b)	0·25	0	0·13	—	0	0	0	0·06
				(c)	0·25	0·44	0·05	—	0	0	0	0·12
$m\sigma\rho$*	6	35	0	(a)	0·28	0·54	0·79	—	0·96	0·92	1·00	0·75
				(b)	0·53	0·33	0·10	—	0·04	0·08	0	0·18
				(c)	0·19	0·13	0·11	—	0	0	0	0·07
$f\sigma\rho$*	6	36	0	(a)	0·36	0·61	0·90	—	0·92	0·92	1·00	0·79
				(b)	0·19	0·10	0	—	0·04	0	0	0·07
				(c)	0·45	0·29	0·10	—	0·04	0·08	0	0·14
$m\epsilon\sigma\tau$*	2	37	2	(a)	0·17	0·25	0·63	—	0·75	0·75	0·50	0·51
				(b)	0·33	0·31	0·13	—	0·25	0	0	0·17
				(c)	0·50	0·44	0·24	—	0	0·25	0·50	0·32
$f\epsilon\sigma\tau$*	2	25	1·5	(a)	0·50	0·50	0·69	—	0·63	0·75	0·75	0·64
				(b)	0·25	0·19	0·19	—	0·12	0·25	0·25	0·21
				(c)	0·25	0·31	0·12	—	0·25	0	0	0·15

For various reasons the experimenters did not act as subjects in this experiment.

* Subject used also in viscosity experiment.

TABLE 3 (a)

	Individual variations	τ	Sex	σ	$\begin{cases}\epsilon \\ \text{age}\end{cases}$	ρ
χ^2	32·6	4·92	2·68	3·58	11·8	15·4
P	$\ll 0{\cdot}01$	0·03	0·10	0·05	$\ll 0{\cdot}01$	$\lll 0{\cdot}01$

TABLE 4

Subject	No. of tests	Method	Doubtful judgments: means per test	Group	1	2	3	4	5	6	7
mσρ	2	A	0	(*a*)	0·25	0·69	0·57	—	0·87	1·00	1·00
				(*b*)	0·33	0·13	0·31	—	0·13	0	0
				(*c*)	0·42	0·18	0·12	—	0	0	0
	3	B	0·3	(*a*)	0·39	0·46	0·54	—	0·92	0·84	0·84
				(*b*)	0·44	0·37	0·33	—	0·08	0·16	0·16
				(*c*)	0·17	0·17	0·13	—	0	0	0
fσρ	2	A	0	(*a*)	0·33	0·75	0·81	—	0·87	1·00	1·00
				(*b*)	0·09	0·06	0·06	—	0·13	0	0
				(*c*)	0·58	0·19	0·13	—	0	0	0
	2	B	0	(*a*)	0·75	0·69	0·88	—	1·00	0·75	1·00
				(*b*)	0	0	0	—	0	0	0
				(*c*)	0·25	0·31	0·12	—	0	0·25	0

Methods A and B were given in a partially randomized order to eliminate any possibility of practice effects influencing the results, although practice effects are singularly lacking in all these experiments.

Scientific training gives a just insignificant superiority, whereas there is definite superiority in the case of the routine testers, and the younger or less educated subjects. The two technologists tested who were well above the average age were significantly inferior judging from the comparatively small number of data available.

That the average "threshold" for compression modulus is about three times as small as that for viscosity, in comparing results from a viscous bitumen and an approximately elastic rubber. The effects of working under water and of not being allowed to feel the sharp edges of the cylinder were very slight. The two subjects tested differed very little from each other in either test. One of them was quite unaffected by the restrictions, in fact her score slightly improved, whereas the other suffered from muscular fatigue, with consequent slight fall in efficiency. These differences in the technique in the viscosity and modulus experiments are therefore quite inadequate to account for the threefold difference in threshold.

Three other minor points are worth noting:

(*a*) Subject *fσρ* was on one occasion not very well and very depressed when she carried out a test. This did not lower her efficiency, but increased her natural time to about double its normal value. This test was not included in the table.

(*b*) Subject *mεσ* took a very detailed interest in the methods of his testing. The fact that he did not prove an efficient subject confirms

Bentley's view (1934) that a "passive set" is advantageous in psychophysical experiments.

(c) Subject *fe* showed a marked fall in efficiency on one occasion when she subsequently reported being emotionally disturbed.

These facts emphasize the probable importance of temporary mental states on the capacity of individuals to judge rheological properties of materials.

4. DISCUSSION

There has been much controversy as to the best way in which to express "thresholds" in psychophysical experiments. It is not intended to participate unnecessarily in these discussions. The whole table of data is given for each experiment. The functional relation between ratio of viscosities or moduli for a pair, and the proportion of correct judgments, is a complex one, and cannot be expressed by a single figure; nor does there seem to be any advantage in this case in choosing an arbitrary point on the curve connecting them and naming it a "threshold". In our experiments, it would not have been advisable to force subjects to select the softer of two samples in cases where they did not feel competent to do so, since such a course would divorce the experiment completely from the practical problems which it was designed to elucidate. We wish to suggest, however, that the best single curve to use to describe the phenomena is that obtained by plotting percentage correct judgments against percentage difference in viscosity or modulus in the pair (Δ).

It happens that, over the range investigated, the curves plotting log Δ against percentage correct are very approximately linear; more nearly so in the case of modulus than viscosity. Further experiment will doubtless show whether this relation has any fundamental significance. Meanwhile, curves so plotted (figure 3) for viscosity and compression modulus would very closely superimpose if the values of Δ for modulus were multiplied by a simple numerical factor in the neighbourhood of three. It would thus be possible to describe the percentage correct/log Δ curve in terms of two parameters, and since one of these, namely that representing the slope of the logarithmic curve, is almost identical for viscosity and modulus, an intercept parameter might be used as a measure of "threshold". We do not, however, consider that the number of data justifies any theoretical conclusions along these lines.

The difference in "threshold" for viscosity and compression modulus is so striking that one is impelled to seek a cause. In a material such as bitumen, if it is desired to keep a constant pressure on the sample, a continuous movement is necessary, whereas for rubber, once the required stress has been reached, the sample could be held at this stress for an indefinite period without further movement. Even if subjects do not actually test the samples in this way (and in fact they do not), this difference of behaviour illustrates a fundamental difference in the reaction of the materials to handling, which will manifest itself whatever method is used by the subject. The viscous materials can only be judged dynamically, whereas the elastic samples can be judged statically.*

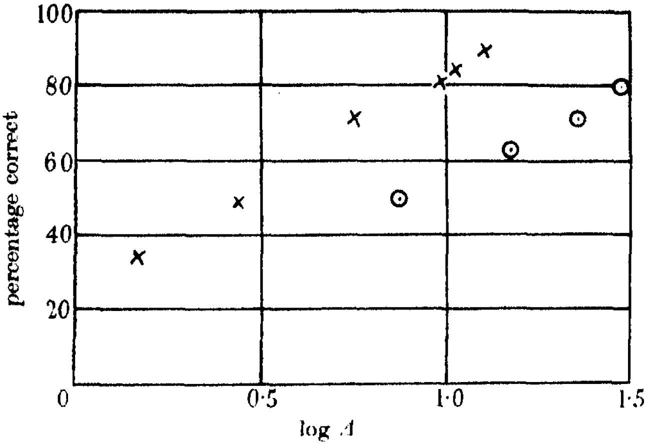

FIGURE 3. × compression nucleus; ⊙ viscosity.

This inherent difference between viscous and elastic materials suggests that the laws relating "threshold" to absolute viscosity and to modulus may not be of the same type. It might be possible to investigate this further by comparing "thresholds" for widely different ranges of viscosity, but the practical difficulties involved in handling the samples are considerable. These matters will be further considered.

In practice, different subjects will apply stresses at different rates both for elastic and viscous materials, and Grindley (1936) has shown that the rate of application of the stimulus may have a great effect on the range of the threshold in the case of pressure. These differences are, however, uncontrollable, and it is hard to see how they can account for the marked differences in "threshold" between viscosity and compression modulus.

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* In comparing two materials showing both elastic and plastic properties in different proportions, one might appear softer than the other if the stress were applied for a long time, whereas the order would be inverted if the stress were applied for a short time. This can readily be shown by comparing a piece of bitumen directly with a sample of rubber. Further, in the case of materials where viscosity changes with stress variation, samples will be relatively softer or firmer depending on the stresses used to deform them.

We are indebted to Professor Bartlett for drawing our attention to these points, and also for other suggestions which have led us to the following further conclusions:

In the first place, it appears that the amount of variability in the sets of judgments is decidedly less than might have been expected. This suggests that factors other than those concerned in the response to the particular tactile qualities are involved, and very quickly became of great importance. We are forced to conclude then, that the expert probably excels not by reason of any superior tactile sense,* but as a result of a combination of a knowledge of how to utilize the information obtained with some other factor. This factor involves the recognition of a functional standard in a way reminiscent of the supposed working of postural schemata (Head, *vide* Bartlett 1932). It is our intention to follow up these suggestions, and at the same time to develop the treatment proposed at the beginning of this paper. The physicist has frequently tried to fit psychological concepts into systems which, although convenient dimensionally, involve operations as drastic as those of Procrustes. In the course of this work the reverse process will be followed. The psychological concepts will be isolated as far as possible and those physical properties utilized which correspond most directly to them; but it was clearly necessary first to determine the "thresholds" for the extreme cases of true fluid and elastic solid as a preliminary to the investigation of more complex systems.

SUMMARY

1. It is suggested that the firmness and "elasticity" (in the colloquial sense of the word) of materials which are neither true fluids nor perfectly elastic solids may be conveniently described by an equation of the type:

$$\log S = \log \psi + \log \sigma - k \log t,$$

where S = stress, σ = strain, t = time, and ψ and k are parameters representing firmness and "elasticity" respectively.

2. As a preliminary to investigating the capacity of skilled craftsmen and unskilled individuals to judge firmness and "elasticity" by means of the senses, "differential thresholds" were measured for the viscosity of a true fluid and the compression modulus of an approximately elastic solid.

3. In the case of viscosity, technological experience and scientific education do not affect the sensitivity of the subject, nor is there much

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* Similar conclusions were drawn in connexion with the judging of wool tops (Binns 1938).

improvement with practice; age and general education appear disadvantageous. With modulus, there were specific differences between subjects, and these were in some cases related to training. The effects of age, sex, etc. are discussed.

4. Subjects can judge differences in compression modulus three times as small as those which can be distinguished for viscosity. Differences of the order of 9 % in the case of modulus and 30 % in the case of viscosity can be detected with some measure of certainty (80 % correct). The use of a single point "threshold" is, however, not recommended; although it is found that a simple equation involving two parameters fits the data obtained. This equation must be regarded as provisional and empirical.

5. Since the superior capacity of experts appears to depend on no greater sensory acuity for the properties investigated than that of other subjects, and since there is not much improvement in this acuity with practice, it seems likely that some type of functional standard is developed which improves the capacity for recognition of rheological conditions. To this must be added the advantages of technological knowledge, making possible adequate utilization of the information obtained. It may be that in the case of the more complex rheological properties not yet investigated, these factors are of even greater importance than in the case of viscosity and compression modulus.

The authors are indebted to Professor F. C. Bartlett, F.R.S., to Dr A. Mace and to Mr A. W. Wolters for helpful suggestions on the psychological aspect of this work, and to Dr P. White both for his interest in the physical side and for his help in designing the rheometer now under construction. They are also indebted to the many subjects who have collaborated.

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Evidence of resistance to tumour grafts in the offspring of immunized rats

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Introduction

For many years various experiments have been devised in order to find out whether an acquired characteristic can be inherited, but the results on the whole do not appear to have received general scientific sanction. C. P. Martin and Bradford Hill in recent years have both made surveys of this subject, but from different points of view. Martin (1937), in making a survey of the literature on the subject, points out many of the difficulties which confront the experimentalist and indicates possible causes for the apparent discrepancies in results. He also raises the possible existence of a temporary form of inheritance which obeys the Mendelian laws. Hill (1934) reviews a great deal of the work which has been done on the possibility of resistance to bacterial infection being inherited. He points out that much of this work is unconvincing, as it is very difficult in the case of bacterial infections to be quite sure that the offspring of the experimental animals have not been infected to some extent, as well as the parents.

The experiments recorded in this paper have resulted from observations made in the course of our work on the propagation of Jensen's rat sarcoma. This tumour is easily transplanted from one rat to another by subcutaneous inoculation of small grafts, and when a sensitive breed of rat is used the tumour very rarely retrogresses, provided a scrupulous technique is followed (Chambers and Scott 1930). Many years ago we noticed that the offspring of rats in which tumours had disappeared were not so susceptible to the tumour grafts as other rats of the same breed, and it seemed worth while to find out how far this apparently acquired characteristic was transmitted to the offspring. The data now recorded provide evidence in support of the view that this acquired characteristic is transmitted to the offspring to some extent.

TECHNIQUE

It is well known that different breeds of rats vary very much in their susceptibility to Jensen's rat sarcoma. In some breeds as many as 70% of the transplants may disappear, while in a susceptible breed the tumour will prove fatal in over 90% of the rats inoculated when very quickly growing tumour is used.

For over twelve years we have used a black and white breed of rat which is very susceptible to this tumour. If small pieces of rapidly growing Jensen's rat sarcoma are inoculated into young rats of this breed about 95% will have progressive tumours and these will eventually prove fatal; actually the rats are killed when the tumours become uncomfortably large. This percentage of progressive tumours may fall to below 90% if rather older rats are used; on the other hand, over 100 young rats about 100 g. in weight have been inoculated in consecutive groups and not one tumour has disappeared. In all the experiments recorded here this breed of rat has been used.

Our stock has not been crossed with any other breed, and if the rats used are about the same age and weight the results of inoculation are consistent, when rapidly growing tumour is inoculated and the greatest care is paid to the general technique. This technique among other things involves the cutting of tumour tissue into small pieces as nearly as possible the same size, because it is found that, within restricted limits, the larger the actual graft the larger the resulting tumour, provided that the other factors controlling growth are kept constant. Out of the 557 control rats of all ages (most of them just over 100 g. in weight) used consecutively just before the experiments given in this paper were begun, forty-seven tumours disappeared, and this 8% disappearance is a very fair average figure.

THE PRODUCTION OF IMMUNITY

Rats become immune to Jensen's rat sarcoma by absorbing the tumour. This may happen in various ways:

(1) A susceptible rat growing a tumour can have the tumour exposed to a lethal dose of X-rays, and if this is accurately calculated the tumour is in most cases gradually absorbed, and the rat is immune to any more inoculations of this tumour (Chambers, Scott and Russ 1922; Russ, Chambers and Scott 1921).

(2) It is possible to produce immunity to Jensen's rat sarcoma by inoculating rats with small pieces of tumour which has been exposed in a dish to a lethal dose of x-rays (Chambers *et al.* 1922; Russ and Scott 1927).

(3) Another and perhaps the easiest way of making a rat immune to Jensen's rat sarcoma is to choose a slowly growing tumour for inoculation. If the less actively growing parts of this are used for transplanting, the majority of the grafts will grow for a little while and then disappear. The rats which have absorbed these slowly growing tumours are generally immune to any more inoculations of Jensen's rat sarcoma (Chambers and Scott 1930).

(4) The immunity may be the result of faults in inoculation technique. If pieces of the tumour are damaged before being inoculated, or are put in such a position that the graft does not get its circulation quickly established, they will eventually be absorbed, and as a rule the rat is afterwards immune (Chambers and Scott 1930).

This immunity, once established by any of these four methods, appears to be equally satisfactory, and to last for the rest of the animal's life (Russ and Scott 1927). In the experiments which follow, one or sometimes several of methods 1–4 have been used in order to produce the immune condition.

EXPERIMENTS TO TEST RESISTANCE OF OFFSPRING OF IMMUNIZED RATS

Four different groups of experiments have been carried out to test whether the offspring of immunized rats were more resistant to tumour growth than those of the same untreated normal stock.

Experiment 1. Rats which had been immunized by absorption of Jensen's rat sarcoma were afterwards mated and their offspring were inoculated at the same time as the offspring of controls.

Experiment 2. Immune male rats were mated to normal stock female rats and their offspring tested in the same way as in experiment 1.

Experiment 3. Normal pairs of stock rats were mated and their offspring tested to see if they were of normal susceptibility. When this had been proved the parents were immunized and afterwards remated. The susceptibility of the offspring born after the parents had been immunized was compared with those born when the parents were normal.

Experiment 4. Two litters of stock rats were chosen and half the males and half the females in each family immunized. When their immunity to Jensen's rat sarcoma had been proved the immune males in one family were mated to the immune females in the other, and vice versa. At the same time the untreated rats in one family were mated to the untreated rats in the other. The offspring of the immune parents were inoculated with Jensen's rat sarcoma and their double-first cousins, the offspring of untreated rats, used as controls (both the experimental and control rats having the same four grandparents).

EXPERIMENTAL METHODS

For every young rat, the offspring of immune parents, a control was chosen from stock of the same sex and of approximately the same weight. The rats were inoculated in pairs—an experimental and its control—with very small pieces of Jensen's rat sarcoma from a rapidly growing tumour. A minute piece of tumour was cut in two under a magnifying glass, the two pieces were made the same size and shape as nearly as possible and then inoculated, one into the experimental rat and one into the control. Measurements of the size of the transplanted tumours were taken twice a week with dividers and a millimetre scale.

In some cases, in spite of great care when inoculating, the small pieces broke and afterwards two or three separate tumours grew. In these cases the total volume of all the tumours per rat has been given, and in most cases this is quite satisfactory, as if the piece broke in one of the pair it generally did in the other; but there are a few exceptions in which it broke in only one. The same sized piece of tumour broken into two or three pieces is apt to give a greater total volume than if it remains unbroken and grows into one large tumour. As a rule the more rapidly growing the tumour the more easily broken it is.

Experiment 1

Table 1 gives in chronological order the results of a series of experiments on a total of 370 rats (185 experimental and 185 control); one column gives the comparative volumes of the tumours in the offspring of the immune rats and in the same number of controls; and in another column the number of tumours in each group which disappeared is given. The volumes of the tumours are given after 17–21 days' growth; after that time some of the control tumours were usually so large that the rats had to be killed. The data in table 1 show that the average volume of the tumours in the offspring of the immune rats was smaller than that of the controls, 30 c.c. compared with 51 c.c., and the number of disappearing tumours was 39 % against 10·8 % in the controls.

The litters of immunized parents varied in the amount of resistance which they showed to the tumour. Some failed to show any appreciable resistance to the growth of the tumour, while others were practically immune, but there has never appeared to be any naturally resistant strain in the normal stock, and the rats which were immunized were taken from

TABLE 1. OFFSPRING OF IMMUNE FEMALES AND IMMUNE MALES

No. of rats in litter	No. of tumours eventually disappearing		Average volume of tumours (c.c.)		Age of tumours days
	Experimental	Control	Experimental	Control	
7	4	1	3·9	20·2	20
6	2	0	41·4	78·5	20
3	1	0	57·8	76·7	21
5	0	0	87·7	76·2	20
6	2	0	51·9	76·2	20
6	1	0	27·4	83·9	19
4	0	0	33·8	83·9	19
8	1	1	32·2	45·1	17
6	1	0	28·2	45·1	17
4	1	2	17·0	43·0	20
6	1	2	21·6	32·8	20
5	5	1	5·4	32·8	20
1	1	0	18·7	28·4	21
5	2	1	27·0	45·6	21
5	1	0	24·2	52·1	18
4	3	1	25·1	33·8	18
10	8	1	10·2	31·8	17
8	4	0	22·8	53·2	17
6	6	0	18·5	79·6	17
2	2	0	6·6	36·6	17
4	0	0	28·8	47·8	17
7	4	1	11·2	41·3	17
7	2	2	43·4	45·1	17
10	0	1	57·2	56·6	17
2	1	0	39·0	43·6	18
5	2	1	21·6	40·2	18
4	1	0	45·8	48·6	18
3	0	0	78·8	42·0	18
5	1	0	42·4	60·0	18
5	1	0	28·4	31·5	18
2	0	1	70·5	38·2	20
1	0	0	6·3	58·8	20
2	0	1	18·0	48·3	20
4	0	0	29·6	24·6	20
1	1	0	20·4	16·5	20
1	1	0	0·0	23·1	21
1	1	0	0·0	12·9	21
1	0	1	42·8	36·3	21
5	5	1	5·8	39·0	21
5	5	1	14·8	96·8	21
3	1	0	22·2	107·0	21
185	72 39 %	20 10·8 %	Av. vol. per rat 30 c.c.	Av. vol. per rat 51 c.c.	

An equal number of control rats was inoculated with each litter.

various branches of the breed. As the young rats used as controls were also picked from a large number of different families there seems to be no reason to assume that those rats immunized came from a less susceptible strain than the parents of the controls against which their offspring were tested.

These experiments suggested that some resistance, though not complete immunity, to Jensen's rat sarcoma was transmitted to the offspring of immunized rats, but there was no light thrown on the point as to whether it was merely a "passive" immunity transmitted by the female parent or whether it could also be handed on by the male.

Experiment 2

To throw some light on this point, male immune rats were mated to stock female rats and their offspring were tested for resistance to Jensen's rat sarcoma. The result of the test inoculations is given in table 2, and shows that, although perhaps the resistance of the offspring to tumour growth was not quite so marked as when both parents were immune, these young rats showed more than the normal amount of resistance. Out of forty-one rats, the offspring of immune male parents, thirteen had disappearing tumours (31·7 %), while three out of the forty-one control rats' tumours disappeared (7·3 %). The average volume of the tumours in the offspring of an immune male parent 17–21 days after inoculation was 28·0 c.c. and that of the controls was 43·0 c.c.

TABLE 2. OFFSPRING OF NORMAL FEMALE AND IMMUNE MALE

No. of rats in litter	No. of tumours eventually disappearing		Average volume of tumours c.c.		Age of tumours days
	Experimental	Control	Experimental	Control	
3	0	0	30·0	37·8	17
3	1	1	28·2	19·3	17
3	0	0	32·0	48·8	17
2	1	0	33·0	67·5	20
2	1	0	20·9	27·2	20
3	1	0	33·8	31·0	18
1	1	0	8·0	93·0	20
2	2	0	1·3	58·7	21
7	2	0	49·2	58·0	21
6	1	1	14·5	38·2	17
4	1	0	21·5	35·6	17
1	0	0	22·2	36·8	17
2	0	0	30·8	31·4	17
2	2	1	31·0	37·0	17
41	13	3	Av. vol. per rat 28 c.c.	Av. vol. per rat 43 c.c.	
	31·7 %	7·3 %			

Experiment 3

The offspring of pairs of normal rats were tested for their susceptibility to Jensen's rat sarcoma; the parents were then immunized, and after their immunity was established they were remated. The resistance of the offspring born *after* their parents had been immunized was then tested, and a comparison made of the resistance to the growth of the tumour in the two batches of offspring, one born before and the others after the parents had been immunized. Ten pairs of young normal stock rats were mated, and their offspring, when about 100 g. in weight, were inoculated with Jensen's rat sarcoma at the same time as an equal number of stock rats of the same sex and weight drawn from various litters. The results showed that the offspring of the specially selected pairs were of normal susceptibility to the tumour. Some of the litters had smaller and some had larger tumours than the average control.

The parent rats were then immunized by treating them with irradiated Jensen's rat sarcoma, and when they had been proved immune to the tumour they were remated to their same mates, and their offspring once more tested for their susceptibility to Jensen's rat sarcoma. These young rats were inoculated in the usual way in pairs with controls of the same sex and weight. After approximately 3 weeks' growth the average volume of tumour was smaller than the controls in all the fourteen litters tested; the average volume was under half (0·44) that of the controls. Table 3 shows the comparison of these results of inoculation of the litters born before and after the parents were immunized.

TABLE 3. DIFFERENCE IN THE RESISTANCE TO JENSEN'S RAT SARCOMA OF OFFSPRING BORN BEFORE AND AFTER PARENTS HAVE BEEN IMMUNIZED

No. of litters of *immune parents	No. of litters in which average volume of tumours was smaller than controls	Average volume of tumours compared with	
		(1) Tumours in controls	(2) Tumours in litters born before parents were immunized
15 (70 rats)	15	0·44	0·50

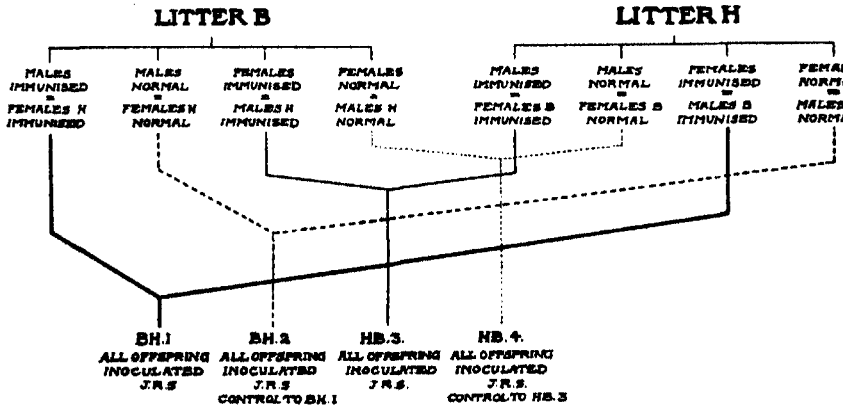
Thirty out of seventy of the tumours growing in the offspring of immune rats disappeared—43 %
Six out of sixty-six of the tumours growing in the offspring of normal rats disappeared—7·6 %

* The offspring born before these parents were immunized were of normal susceptibility.

These results indicated, without much doubt, that rats born after their parents had been immunized to Jensen's rat sarcoma were much more resistant to that tumour than the litters born before the parents were treated.

Experiment 4

Comparative susceptibility of double-first cousins. Litters of young stock rats were taken, and half the males and half the females in each litter immunized to Jensen's rat sarcoma. When the treated rats had been definitely proved to be immune to Jensen's rat sarcoma, the immune rats in one family (B) were mated to the immune rats in another family (H) at the same time as the normal (B) males and females were mated to the normal (H) males and females. This was done with a number of pairs of families. In this way any offspring born to the B and H rats would have the same four grandparents, the one great difference being that some would be the offspring of immune parents and some the offspring of normal rats. A typical family tree is given in figure 1. When the offspring of the immune parents were young adults their resistance to Jensen's rat sarcoma was tested at the same time as their control double-first cousins (i.e. those rats with the same four grandparents). A certain number of control rats from the ordinary stock were always inoculated as extra controls; these are not included in the tables.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BH.2 ALL OFFSPRING INOCULATED J.R.S CONTROL TO BH.1
HB.3. ALL OFFSPRING INOCULATED J.R.S.
HB.4. ALL OFFSPRING INOCULATED J.R.S. CONTROL TO HB.3

FIGURE 1. Family tree of rats. Double-first cousin experiment.

Seventeen litters, the offspring of immune parents, have been inoculated with Jensen's rat sarcoma at the same time as their double-first cousins. In every case but one the average size of the tumours in the litters of the immunized rats was smaller than the average tumours in their double-first cousins used as controls. As there were no cousins available for two extra litters of immunized parents, ordinary control rats were used (see table 4).

TABLE 4

Tumours of offspring of immunized rats Inoculated at same time as normal double-first cousins			Tumours in offspring of normal rats Double-first cousins of experimental rats (in first column)		
No. of rats in litter inoculated	Average volume of tumour 18–21 days after inoculation c.c.	No. of tumours which disappeared	No. of rats in litter inoculated	Average volume of tumour 18–21 days after inoculation c.c.	No. of tumours which disappeared
Group 1					
6	7·1	5 out of 6	3	35·2	1 out of 3
7	14·8	3 „ 7	5	42·8	1 „ 5
3	32·8	1 „ 3	1	41·8	1 „ 1
9	53·0	1 „ 9	4	42·4	1 „ 4
Group 2					
3	2·8	3 out of 3	3	28·6	0 out of 3
3	0·1	3 „ 3	5	25·4	0 „ 5
6	30·3	5 „ 6	3	68·8	0 „ 3
Group 3					
1	25·4	0 out of 1	1	202·0	0 out of 1
6	40·4	1 „ 6	6	110·0	0 „ 6
2	8·4	2 „ 2	1	136·0	0 „ 1
3	65·0	0 „ 3	2	108·0	0 „ 2
4	8·1	4 „ 4	2	82·8	0 „ 2
1	33·4	0 „ 1	2	52·4	1 „ 2
10	14·7	8 „ 10	2	164·0	0 „ 2
3	1·4	3 „ 3	2	180·0	0 „ 2
Group 4					
10	30·0	These tumours all excised for alternative measurement (see text)	5	64·6	These tumours all excised for alternative measurement (see text)
5	61·7		6	144·6	
3	37·4		*4	113·0	
4	33·8		*4	58·0	
Total	Average volume per rat	39 out of 67	Total	Average volume per rat	5 out of 42
89	29·8 c.c.	58·2 %	61	74·5 c.c.	11·9 %

* These are *not* first cousins of experimental rats.

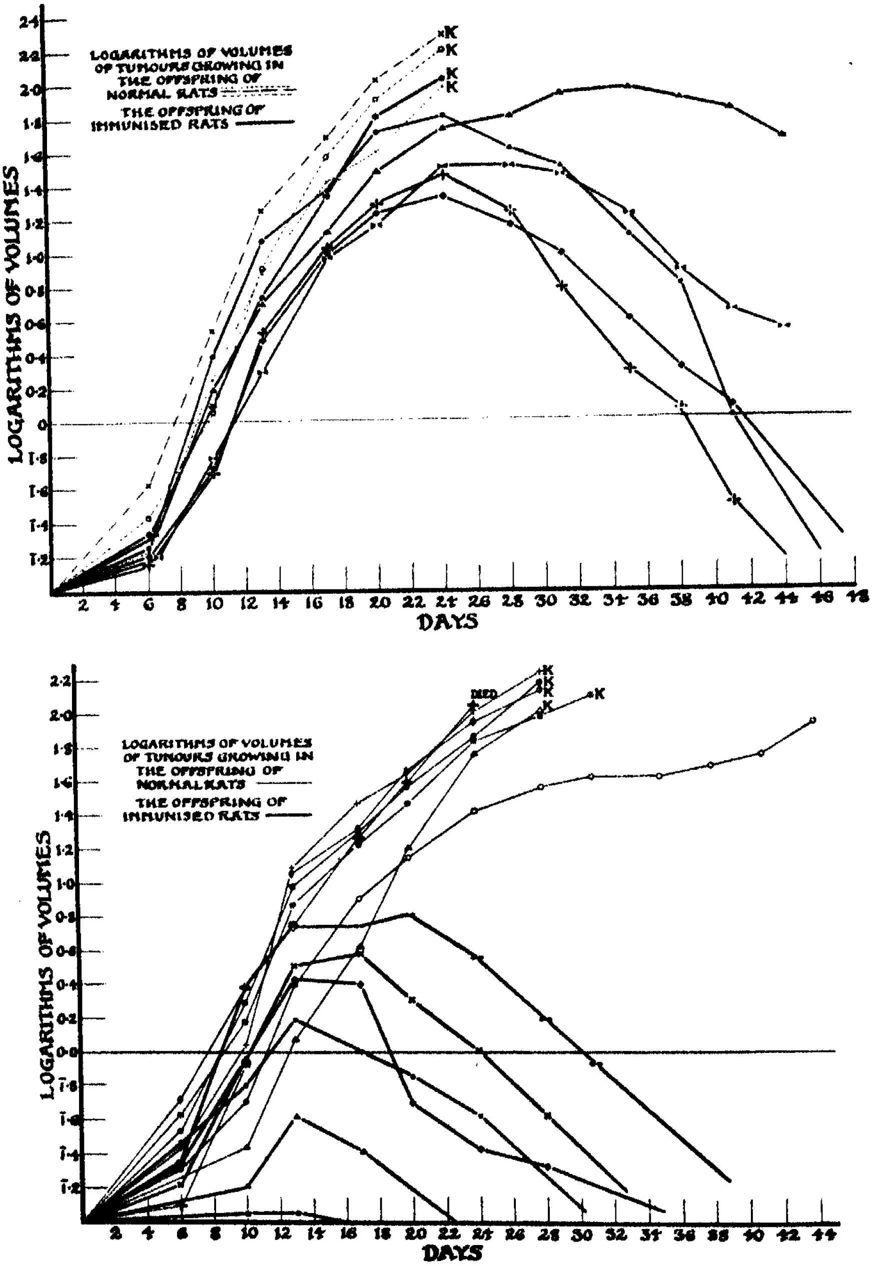
The results of the inoculation of eighty-nine experimental rats which were the offspring of immune parents are given in table 4, and show that the average volume of the tumours 18–21 days after inoculation was 29·8 c.c. compared with 74·5 c.c. in the controls which were their double-first cousins. The number of tumours disappearing was 58·2 % compared with 11·9 % in the controls. The tumours in the last group of the table could not be included in the percentages of disappearing tumours as they were all excised in order to check the comparative volumes of the tumours in the experimental rats and controls by another method: each tumour was excised and the displacement in water measured. By this method the volume of the experimental tumours was found to be 0·44 that of the control. When the tumours had been measured in the ordinary way with dividers and a millimetre scale earlier in the day while still in the rat the volume ratio calculated was 0·40 that of the controls.

Dotted lines have been drawn across the table marking the division between the four different groups of rats inoculated with different tumours. All the rats in any one division were inoculated with the same tumour. Two graphs of the logarithmic rate of growth of the tumours in group 2 of this table are given in figure 2.

Note added October 1939. During the last six months some experiments have been carried out to see whether this inherited resistance to inoculation of Jensen's rat sarcoma is transmitted to the next generation.

Six litters, the offspring of untreated parents but the grandchildren of immunized rats, were inoculated with Jensen's rat sarcoma at the same time as their control cousins who had untreated grandparents as well as parents. At the end of three weeks (approximately) the tumours in the descendants of the immunized grandparents averaged under half the volume of those growing in the controls. A table of these results is given below. These figures show no obvious falling off from the amount of resistance transmitted to the first generation (see table 4).

Litter pairs	No. of rats in litter	Average volume of tumour in grandchildren of immunized rats	Average volume of tumour in control cousins (grandchildren of normal rats)
		c.c.	c.c.
1st	8	17·0	48·8
2nd	5	23·4	41·6
3rd	6	48·3	128·5
4th	7	69·6	98·0
5th	3	42·6	104·4
6th	4	17·8	113·0



——— = Experimental } Experiments 5 and 6 in table 4.
–·–·–· = Controls

——— = Experimental } Experiment 7 in table 4.
- - - - - = Controls

FIGURE 2. Rates of growth of tumours growing in rats, the offspring of immunized parents, and in their double-first cousins, the offspring of normal rats.

SUMMARY OF RESULTS

If the results of these groups of experiments are considered together, the average volume of the Jensen's rat sarcoma tumours in 385 rats, the offspring of immunized parents, is just under half that of the controls 17–21 days after inoculation when most carefully controlled (see tables 3 and 4), and just over half the volume when only the male parent has been immunized (see table 2).

The number of disappearing tumours was much greater in the offspring of the immunized rats, 42·0% disappearing compared with 10·0% in the controls.

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The effect of subnormal temperature on the differentiation and survival of cultivated *in vitro* embryonic and infantile rat and mouse ovaries*

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[Plates 8, 9]

Introduction

In previous experiments (1937, 1938 *a*, *b*) it was shown that embryonic and infantile rat and mouse ovaries survive *in vitro* for several weeks and that the germ cells continue their normal differentiation. After about 28 days' cultivation, however, the explanted ovaries began to degenerate and in a few days became hopelessly necrotic.

Recently the effect of incubating such explants at a subnormal temperature has been investigated and a remarkable improvement in the health and longevity of the cultures was obtained. In the present communication the behaviour of the ovary under these conditions of cultivation is described and the significance of the results in relation to the theory of the storage of germ cells is considered.

A summary of the relevant literature has been given in the previous communication (1938*a*).

Material and methods

The ovaries were obtained from mice ranging in age from the 15th–16th day of embryonic life to the 7th day after birth and from rats ranging in age from the 18th day of foetal life to the 3rd day after birth.

The culture technique differed from that previously described (1938*a*) in only two respects. A number of the cultures were incubated at 34° C instead of at 37° C, and the interval between subcultures was prolonged from 3 days, as in the previous experiments, to 7–14 days. It was found that too frequent changing of the culture medium caused cumulative

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damage to the explant resulting in premature death. By prolonging the interval, better and more prolonged growth was obtained even at 37° C.

The numbers of cultures studied are tabulated below.

	Duration of culture period days	No. of cultures at 34° C	No. of controls at 37° C	No. of cultures grown first at 37° C and later at 34° C
Mouse	1– 28	15	15	—
,,	28– 80	12	2	6
Rat	1– 28	5	5	—
,,	28–103	2	3	6

The histological technique was the same as that previously described.

RESULTS

Explants of the mouse ovary incubated at 34° C

During the first month *in vitro* there was little difference between the ovaries incubated at 34° C and the controls incubated at 37° C except that the former liquefied the culture medium less than the latter. The lower temperature depressed somewhat the growth of the explant but not the rate of differentiation of the germ cells which passed through the various stages of meiosis at the same time as in the controls. As in the earlier experiments (figure 2, plate 8), the germinal peritoneum disappeared completely in both experimental and control cultures after about a week *in vitro*.

Relays of a few ova at a time enlarged, attained full size and finally degenerated. Sometimes the enlarged ova reached the metaphase of the first maturation division but were not seen to develop beyond this stage. The frequency with which the metaphase was observed in the full-grown ova even to the exclusion of the preceding prophase, suggested that this phase must be of unusually long duration.

The ovaries from the 7-day mice already contained growing ova at the time of explantation. These continued to enlarge *in vitro* and even showed maturation divisions, indicating that the growing ovum is less sensitive to environmental disturbance than is sometimes supposed.

In my earlier experiments no zona pellucida was observed around the enlarging ova, but in the cultures now being described some at least of the growing ova in both the experimental and control explants were invested by a distinct hyaline membrane probably identical with the zona pellucida. This zone, which varied in thickness in different cells, was sometimes visible in sections, but was seen best in living ova teased out of the surrounding stroma. Most of the growing egg cells were also surrounded by a thin envelope of follicular cells.

As already stated, only a few ova enlarged at a time, and the vast majority remained stored in the ovary for long periods. As in the normal embryo, germ cells might degenerate at any stage in their life-history but they did so most commonly during the early stages of the meiotic prophase.

After the last ova had passed through the early stages of the meiotic prophase the histological appearance of the explants did not materially alter. Relays of ova continued to enlarge and then degenerate, the rest remaining unchanged. No periodicity in the growth of the ova was observed. As in the normal post-embryonic ovary up to the time of puberty, enlarging ova in all stages of development occurred throughout the life of the explant. The number of full-grown ova present at any given time varied from twenty to fifty.

By about the 30th day of incubation, marked differences between the experimental and control explants began to appear. The ovaries incubated at 34° C contained more growing ova and showed less degeneration among the resting ova than those grown at 37° C.

By prolonging the interval between subcultures, as described above, it was sometimes possible to keep control explants fairly healthy at 37° C for 5–6 weeks but the majority degenerated before the 30th day. When degeneration began the resting ova showed marked changes. The nucleus became swollen, the nuclear material stained less intensely and the cytoplasm had a ragged appearance especially at the periphery. If incubation at 37° C was continued (figure 4, plate 8), the explant became opaque, growth of the ova ceased and the culture became very necrotic. If, however, the unhealthy explant was transferred to an incubator at 34° C, it recovered. The oocytes gradually resumed their healthy appearance and began to enlarge once more.

Mouse ovaries kept at 34° C remained in a comparatively healthy state for as long as 70–80 days. Maturation figures have been observed 2 months after explantation (figure 5, plate 9). The explants eventually disapeared, not by a wholesale necrosis of the entire organ as in the controls, but by a progressive diminution in size which accompanied the gradual disappearance of the oocyte reserves. This decrease in size was caused by three factors:

(1) A tendency of the ovary to spread over the plasma clot as a thin sheet of tissue (figure 1, plate 8). In consequence, when the explant was removed from the plasma during subcultivation, many cells were left

behind attached to the clot. At the same time many ova of various sizes escaped and were lost.

(2) Continuous degeneration of the oocytes.

(3) A low growth rate of the stroma. The stromal cells remained quite healthy *in vitro* but showed very few mitotic figures. Thus the tissue lost during subcultivation was not replaced by subsequent growth.

As a result of these three factors, by the 80th day of cultivation the ovary was reduced to a handful of stroma cells containing about a dozen healthy resting ova and about as many large ova in process of degeneration.

A number of embryonic mouse ovaries were incubated at 32° C, but they did not develop so well as those kept at 34° C and showed more symptoms of degeneration. The optimum temperature for the survival of mouse ovaries *in vitro* appears therefore to be about 34° C.

Explants of rat ovary incubated at 34° C

As previously recorded (1938*a*), mouse and rat ovaries develop similarly during the first month *in vitro*, but show certain differences in their habit of growth which in the present experiments caused the rat ovary to survive at 34° C for a much longer time than the mouse ovary.

Thus, unlike the mouse ovary, the explanted rat ovary retains more or less its spherical shape, although it produces a rich outgrowth of cells into the culture medium (fig. 6, plate 9). It was therefore easy to detach the organ from the clot without loss of tissue.

The control rat ovaries at 37° C sometimes survived for 60–70 days, provided the interval between subcultures was prolonged to 5–6 days. Towards the end of this period however, cell division in the stroma diminished, large numbers of stromal and egg cells degenerated and the explants became rapidly smaller and finally died. The ovaries grown at 34° C, on the other hand, showed no signs of regression. The stroma, unlike that of the mouse ovaries at 34° C, continued to grow actively, so that the ovary, instead of diminishing in size, enlarged to several times its original volume. Two cultures were kept in a healthy condition for 103 days (figures 3 and 6, plates 8, 9) and when sectioned, their histological appearance indicated that they could have survived for a much longer period, perhaps indefinitely.

As compared with the mouse ovaries (cf. figures 1, 3, 5 and 6, plates 8, 9), only very few oocytes at a time enlarged to full size in the explanted rat ovary, but more ova enlarged at 34° C than at 37° C. No healthy maturation figures were observed and the full-sized ova often showed degenerative fragmentation of the chromatin. Most of the oocytes in the rat explants were of the resting type. At 34° C many of these resting ova survived in an apparently normal condition for 103 days and to judge from their healthy appearance would probably have lived much longer.

DISCUSSION

Why the explanted mouse and rat ovaries should thrive so much better at a temperature several degrees below normal than they do at ordinary body temperature is not clear. The following very tentative suggestion may be offered. The absence of a blood circulation in tissue cultures implies a slower exchange of metabolites between the tissue and its nutritive medium. If the tissue were metabolizing at its normal rate, this would tend to cause degenerative changes in the cells. It is possible that by lowering the temperature the metabolic rate is reduced and the cells are less seriously affected by the imperfect exchange of metabolites. This explanation is purely hypothetical and requires biochemical verification.

In my previous communication (1938*a*) it was stated that "those who support the view that embryonic ova degenerate early agree that they normally survive for a period which exceeds the maximum culture period in the present experiments. In order to test the storage theory more completely it would be highly desirable, therefore, if a method was found of keeping the resting ova *in vitro* for longer periods of time than it has been possible heretofore". The present experiments have provided this method, and the results prove that ova derived from primitive germ cells can survive in the rat ovary for at least 3½ months, a much longer period than is thought possible by those who do not believe in the storage theory.

My thanks are due to Dr H. B. Fell, Director of the Strangeways Laboratory, for her valuable advice and continual interest in this work, and to Mr V. C. Norfield who is responsible for the photomicrographs.

SUMMARY

1. Embryonic and infantile mouse ovaries incubated at 34° C survived *in vitro* for as long as 80 days and some of the germ cells persisted throughout this period. Control cultures incubated at 37° C never survived longer than 30–40 days.

2. During the first 2 months' cultivation relays of germ cells enlarged, attained the normal maximum size and sometimes passed into the metaphase of the first maturation division. Later stages than this were not observed.

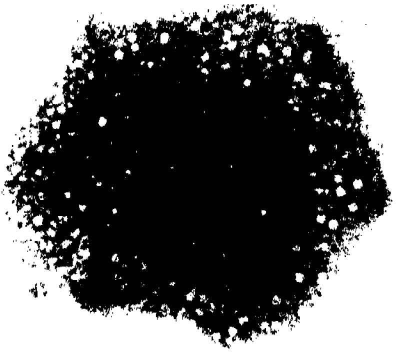

FIGURE 1

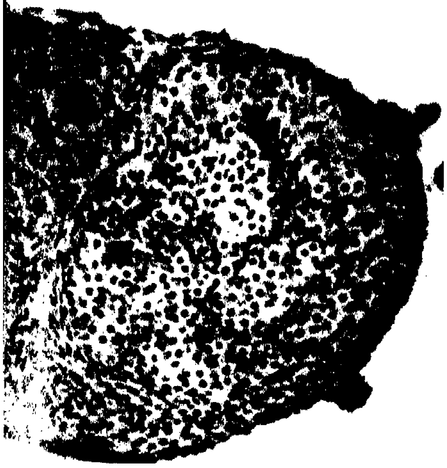

FIGURE 2

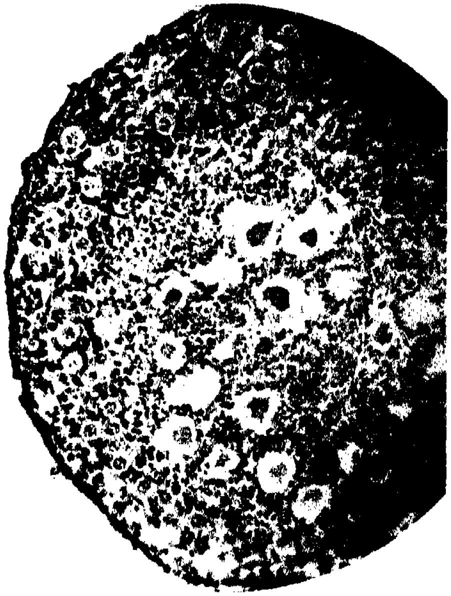

FIGURE 4

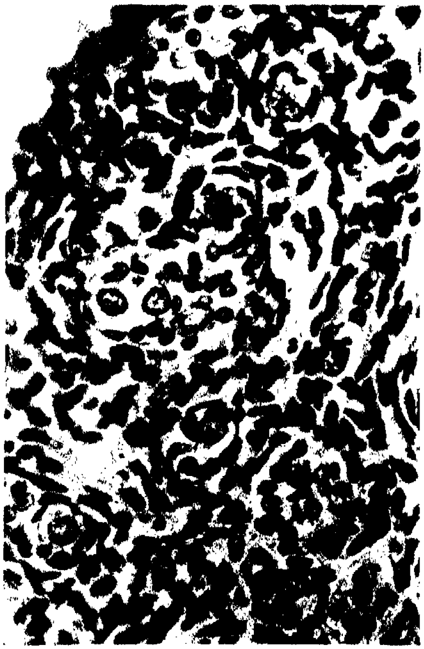

FIGURE 3

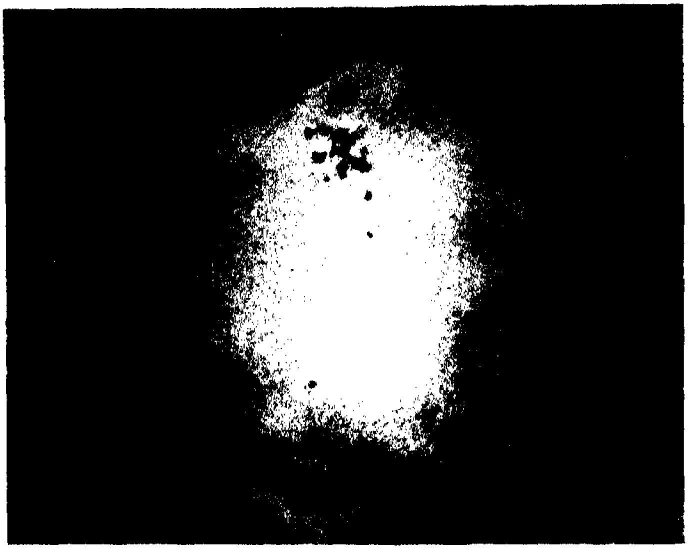

Figure 5

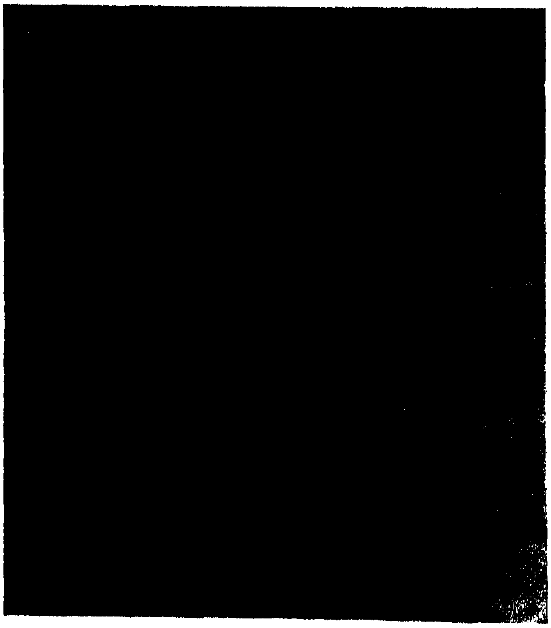

Figure 6

3. Infantile rat ovaries incubated at 34° C were quite healthy after 103 days *in vitro*, whilst controls at 37° C degenerated after 60–70 days.
4. Healthy residual ova were present in cultures of rat ovaries after 103 days *in vitro*.

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DESCRIPTION OF PLATES 8, 9

Figures 1 and 6 are photographs of living cultures, figures 2, 3, 4 and 5 of stained preparations.

FIGURE 1. A living ovary from a 7-day mouse after 39 days' cultivation at 34° C. Note the large number of full-grown ova. × 30.

FIGURE 2. Section through a 15–16 day embryonic mouse ovary cultivated at 34° C for 48 hr. This photograph shows the leptotene and the synaptene phases of meiosis which appeared during cultivation *in vitro*. (Zenker and Feulgen. × 160.)

FIGURE 3. Section through the same explant as that shown in figure 6, fixed after 103 days' cultivation. Note the resting ova surrounded by a few follicle cells.

FIGURE 4. Section through a 4-day mouse ovary after 35 days' cultivation at 37° C. Note the central necrotic area with remnants of the former Graafian follicles. (Zenker; Meyer's haematoxylin. × 180.)

FIGURE 5. Section through the ovary of a 4-day mouse after 58 days' cultivation at 34° C, showing a healthy maturation division figure. (Zenker and Feulgen. × 900.)

FIGURE 6. The living ovary of a 2-day rat after 103 days' cultivation at 34° C. × 20.

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FIGURE 6. The living ovary of a 2-day rat after 103 days' cultivation at 34° C. × 20.

The monaural threshold: the effect of subliminal and audible contralateral and ipsilateral stimuli

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Introduction

Using the experimental technique developed during earlier work on the binaural threshold (Hughes 1938), the effect on the monaural threshold of subliminal notes has now been investigated. It was hoped that the experiments, when carried out with two notes of various frequency differences, would yield information on the extent to which the cochlear resonators postulated by the resonance theory could be influenced by notes of frequencies different from their natural frequencies, and thus permit the determination of the amount of damping to which they were subject. It was anticipated that the threshold of a note of given frequency would be lowered by the simultaneous application to the ear of a second subliminal note provided that the two frequencies were not too far apart. In practice it was found that no matter how different the frequencies, a process of central summation produced such lowering of threshold. The purely physical question of what range of resonators in the cochlea responds to a given exciting tone cannot therefore be investigated in this way.

The lowering of threshold of one ear due to simultaneous subliminal stimulation of the other (Hughes 1938) made it of interest to investigate the case when the note applied to the second ear is audible. Previous workers (e.g. Harvey Fletcher 1929) have shown that when a "masking tone" is well above threshold no change of contralateral threshold is produced. The experimental results described below bear this out in a general way, but the presence of a weak audible tone causes so much difficulty in determining the contralateral threshold that it is not possible to find any critical intensity at which summation ceases. When, however, the two notes were in unison a rise in this threshold occurred, the explanation of which is discussed later.

A tentative investigation of the corresponding monaural problem was made, but no significant results were obtained.

THE APPARATUS

The apparatus and experimental procedure were those of the previous paper. The variable shunt on each telephone was later replaced by a third potentiometer, when a slight rearrangement of the circuit became necessary, and a double-pole double-throw mercury switch rocked by an eccentric on a rotating shaft was substituted for the mercury commutator with rotating vanes used in the earlier work.

1. THE EFFECT ON THE MONAURAL THRESHOLD OF AN IPSILATERAL SUBLIMINAL STIMULUS

The apparatus. For this work various modifications of the original apparatus were made. The first tests were made with the two notes presented to the ear, after electrical summation, in the same telephone; two different forms of mixing circuit were used to combine them. Though electrical summation of the voltages applied to the telephone in these experiments appeared, according to the tests adopted, to be satisfactory, some doubt still existed as to the true nature of the sound output from the telephone under these conditions. The remainder of the experiments were therefore carried out with a different arrangement. Two separate telephones were used in circuits similar to those employed in the work on binaural stimulation; they were mounted side by side, about 6 in. from the ear, in a box lined with absorbent material. The latter had an opening about 3 in. square at the end opposite to the phones, and this was held against the ear. Thus the ear was shielded from any extraneous sounds, and the absorbent lining tended to give, with the low intensities used, the effect of propagation in free space. Any tendency to resonance which might still have been present would not affect the results, since the conditions at the threshold, from which the intensities were measured, would be similarly affected at any definite frequency.

Holes were cut in the box large enough to clear the body of the phone, but smaller than its cap, and a thick ring of sponge rubber was fitted round the phone. A metal plate with a hole in the centre, with a second sponge rubber ring between it and the cap, was bolted to the box so as to hold the phone in the hole between the two thicknesses of sponge rubber. The phones were thus insulated from vibration of the box, and from one another. The lining of the box was of thick cotton-wool, held in place by coarse medicated gauze, and it was continued out of the open end of the box to give a comfortable and close fit to the side of the head of the observer.

For these experiments, the amplifiers used in the binaural work were employed, incorporating three independent intensity controls in each. The 5 db. step-attenuator preceded the first valve, the calibrated dial the second, and the uncalibrated 50,000 ω potentiometer (replacing the original telephone shunts) the third. The experimental procedure was exactly the same as for the previous experiments on similar lines.

After some preliminary results, in which those previously obtained were verified, several sets were taken at unison with a note of 1100 cyc./sec. in each phone, to test if physical summation of the telephone outputs were taking place at the ear. Only one oscillator was used to produce the note, and the two input potentiometers were connected in parallel to its coupling condenser by lead-covered cable with the covering earthed. The voltages applied to the amplifier output circuits were then adjusted to zero phase difference with a cathode-ray oscillograph in the usual way, using a variable air condenser (0·0015 μF max.) connected across the appropriate output circuit to obtain a straight-line trace instead of an ellipse on the oscillograph screen. The corresponding pairs of reductions in intensity necessary when physical summation of the two notes takes place at unison can be calculated as follows: suppose the threshold amplitude is A_0, and is the same for each phone, corresponding to an intensity κA_0^2, where κ is constant under the conditions of any one experiment. A reduction in intensity of x db. in one note reduces the amplitude to A_x where

$$\log_{10}(\kappa A_0^2/\kappa A_x^2) = x/10, \quad \text{or} \quad A_x = A_0/10 \,.\, 10^{(1-x/20)}.$$

If the other note is reduced in intensity by y db., its amplitude becomes $A_y = A_0/10 \,.\, 10^{(1-y/20)}$. Thus to restore the original amplitude A_0 as the sum of the two amplitudes A_x and A_y we must have

$$A_0/10[10^{(1-x/20)} + 10^{(1-y/20)}] = A_0, \quad \text{or} \quad 10^{(1-x/20)} + 10^{(1-y/20)} = 10.$$

Table 1 shows corresponding pairs of values of x and y.

TABLE 1. PAIRS OF VALUES OF x AND y SATISFYING THE RELATION $10^{(1-x/20)} + 10^{(1-y/20)} = 10$

x	4·4	5·1	6·0	7·0	8·0
y	8·0	7·0	6·0	5·1	4·4

Experiments were carried out with reductions from threshold of 5·0, 6·0 and 7·0 db. in the one phone with results for the reduction required in the other indicated in table 2.

TABLE 2. NOTE OF 1100 CYC./SEC. IN EACH TELEPHONE. MEAN OF TEN SETTINGS FOR EACH REDUCTION

		db.	db.	db.
Reductions from threshold	A	5·0	6·0	7·0
	B	−(7·0 ± 0·6)	−(6·3 ± 0·6)	−(5·7 ± 0·6)

They agree very well with the calculations, and indicate that physical summation is fairly complete with the arrangement described.

The main experiments were continued by taking the threshold reductions of one note due to the presence of another subliminal note of the same or different frequency 2·0, 3·0 and 4·0 db. below its threshold at various pairs of frequencies. The results thus obtained, together with those found with the method of electrical mixing, are summarized in table 3. Each result is, as before, the mean of ten settings of the dial.

TABLE 3. REDUCTIONS FROM MONAURAL THRESHOLD INTENSITY

Frequencies of notes		Db. below monaural threshold of note A			
		2·0	3·0	4·0	
A	B	Db. below monaural threshold of note B			Observer
1120	1100	2·4 ± 0·9	1·7 ± 0·8	1·1 ± 0·5	J. W. H.
		1·9 ± 1·3	2·2 ± 0·6	1·9 ± 0·8	
		2·7 ± 0·5	2·4 ± 0·3	1·8 ± 0·5	
1050	1100	0·7 ± 0·4	0·6 ± 0·3	0·5 ± 0·5	
		3·6 ± 1·6	2·8 ± 0·9	1·8 ± 0·9	W. L. B.
1000	1100	0·6 ± 0·5	0·5 ± 0·5	0·4 ± 0·5	J. W. H.
		3·0 ± 1·0	2·2 ± 0·6	1·4 ± 0·5	W. L. B.
900	1100	0·7 ± 0·6	0·6 ± 0·6	0·5 ± 0·4	J. W. H.
700	1100	2·2 ± 0·4	2·1 ± 1·0	2·1 ± 0·3	
		2·4 ± 0·8	2·0 ± 0·8	2·1 ± 0·9	
		1·9 ± 0·6	1·5 ± 0·6	1·1 ± 0·5	
		2·2 ± 0·5	1·8 ± 0·5	1·6 ± 0·5	
		0·6 ± 0·5	0·6 ± 0·6	0·4 ± 0·4	
		2·2 ± 0·4	1·6 ± 0·9	1·6 ± 0·9	W. L. B.
		1·6 ± 1·5	1·2 ± 1·2	1·1 ± 1·0	
300	1100	2·4 ± 0·4	1·9 ± 0·3	1·5 ± 0·8	J. W. H.
250	310	0·6 ± 0·5	0·9 ± 0·7	0·7 ± 0·7	
290	310	1·3 ± 0·5	0·8 ± 0·8	0·7 ± 0·7	
450	470	2·4 ± 1·2	1·7 ± 1·0	2·1 ± 0·6	
	480	1·8 ± 0·9	2·0 ± 1·0	1·4 ± 1·0	
	490	1·4 ± 1·1	1·1 ± 1·0	1·3 ± 1·1	
2900	3000	2·4 ± 1·2	2·0 ± 1·4	1·7 ± 1·0	
2800	3000	2·7 ± 1·7	1·8 ± 1·2	1·7 ± 1·0	
310	2900	1·9 ± 0·9	1·7 ± 0·8	1·3 ± 0·5	
Mean value of $(E_1/E_0)_L + (E_1/E_0)_R$ for all frequencies		1·31	1·22	1·13	J. W. H.
		1·19	1·15	1·11	W. L. B.

It will be seen that some reduction from threshold was always necessary, but that its amount varied considerably, even for one observer at the same pair of frequencies. A second observer (W. L. B.) took a few sets of results, and these were in fair agreement with those of the first (J. W. H.). However, the same order of reduction was required for both high and low frequencies, as the table shows, and even for a pair of notes differing widely in pitch, e.g. 310 and 2900 cyc./sec. This summational effect cannot be due to interaction between cochlear resonators, since it occurs with such wide differences of pitch; it must be central. If the cochlear resonators do act upon each other when their frequencies are close together, the effect cannot be observed owing to this general summation. The pairs of frequencies were chosen, as in the earlier work, to avoid the possibility of common overtones in the audible range. The mean total energy required at the two ears for all pairs of frequencies, expressed for each observer in terms of the monaural threshold energy E_0, is shown in the last two lines of the table. In each case, the sum is greatest, i.e. the summation is least, when the intensity of the subliminal note is greatest, and decreases with the intensity of the subliminal note. The significance of this is discussed later.

2. THE EFFECT ON THE MONAURAL THRESHOLD OF AN IPSILATERAL AUDIBLE STIMULUS

The apparatus used in the previous section was employed for these experiments, and the experimental procedure was unaltered. The audible note was maintained at 2·0, 3·0 and 5·0 db. above its threshold; this was arranged by raising the intensity by 5·0 db. on the step-attenuator, and decreasing it by 3·0 or 2·0 db. on the dial potentiometer to obtain a total rise of 2·0 or 3·0 db. above threshold.

The results were taken with notes of 700 and 1100 cyc./sec., with the 700 cyc./sec. note at one of these fixed intensity levels (2·0, 3·0, or 5·0 db. above threshold). In the first set, with both notes interrupted by the rocking switch previously mentioned, no definite rise or fall in the threshold of the 1100 cyc./sec. note could be found, as the "scatter" among the settings was too large. The observer (J. W. H.) found the audibility of the 1100 cyc./sec. note difficult to judge with a note of 700 cyc./sec. distinctly audible at the same time, and he tended to use the "purity" of the 700 cyc./sec. note as the criterion of audibility. In an attempt to overcome this, the 700 cyc./sec. was made continuous after fixing its intensity at the appropriate level; the muting switch inside the room could be used to prevent fatigue, if that proved necessary. The "scatter" was reduced a little by this method, though it was still large enough to hide any definite threshold change in either of the two observers. In fact, the difficulty of judgement found in the first experiments was still present and no further results were attempted. Those that had already been taken are shown in table 4.

TABLE 4. REDUCTIONS FROM MONAURAL THRESHOLD INTENSITY WITH AN IPSILATERAL AUDIBLE STIMULUS

Frequencies of notes		Db. above monaural threshold of note A			
		2·0	3·0	5·0	
A	B	Db. below monaural threshold of note B			Observer
700 (interrupted)	1100	0·6 ± 2·1	0·7 ± 1·4	0·7 ± 1·9	J. W. H.
700 (continuous)	1100	− (0·8 ± 1·0)	− (0·5 ± 1·9)	− (0·4 ± 1·2)	J. W. H.
700 (continuous)	1100	0·7 ± 1·7	0·7 ± 1·5	1·1 ± 1·8	W. L. B.

3. THE EFFECT ON THE MONAURAL THRESHOLD OF AN AUDIBLE CONTRALATERAL STIMULUS

The apparatus was not changed for this work, except that two telephones were used to convey the sounds to separate ears. The experimental procedure remained unaltered, and the known audible intensities were produced by the combination of settings of two potentiometers described in § 2.

The results obtained are set out in table 5; in all cases, note A was fixed in intensity and made continuous, and the "binaural threshold" was found by varying the intensity of note B.

TABLE 5. REDUCTIONS FROM MONAURAL THRESHOLD INTENSITY IN THE RIGHT EAR, WITH AN AUDIBLE NOTE IN THE LEFT

Frequencies of notes (cyc./sec.)		Db. above monaural threshold in left ear					
		2·0	3·0	5·0	7·0	10·0	15·0
Left ear	Right ear	Db. below monaural threshold in right ear					
700	1100	−0·9 ± 2·7	−1·0 ± 3·8	+0·2 ± 1·9			
				+0·1 ± 1·9	−0·6 ± 2·8	+1·0 ± 2·1	
				+1·2 ± 3·3		+1·0 ± 4·8	−0·7 ± 3·6
1000	1100	−1·1 ± 1·6	−0·9 ± 1·6	−1·0 ± 1·8			
				−0·1 ± 2·8	−0·4 ± 1·9	−0·2 ± 2·9	
				+0·5 ± 2·0		+0·8 ± 2·4	−0·4 ± 2·6

Three sets of three tests each were made with pairs of frequencies 700 and 1100 cyc./sec., and 1000 and 1100 cyc./sec. In the first three, the intensities of the audible note were 2·0, 3·0 and 5·0 db. above threshold; in the next, they were 5·0, 7·0 and 10·0 db. above threshold, and in the last they were 5·0, 10·0, and 15·0 db. above threshold. The increase in the intensities of the audible notes was made when no conclusive results were obtained with those first tried, and even with one note at 15 db. above the threshold in one ear, no definite effect was produced on that of the other in the opposite ear. However, when the frequencies of the notes in the two ears was made the same, an unmistakable rise of threshold became evident (table 6). Though there seemed to the observer (J. W. H.) to be no particular difficulty in deciding whether the note of variable intensity was audible under the conditions of these experiments, the settings have a "scatter" as large as, if not larger than, those in the preceding section, when this difficulty was certainly encountered.

TABLE 6. RISE OF MONAURAL THRESHOLD INTENSITY IN THE RIGHT EAR, WITH AN AUDIBLE NOTE OF THE SAME FREQUENCY (1100 CYC./SEC.) IN THE LEFT

Db. above monaural threshold in left ear		
5·0	10·0	15·0
Db. above monaural threshold in right ear		
4·1 ± 3·2	7·3 ± 3·8	9·5 ± 3·2

DISCUSSION

The results of the investigations described in this and the previous paper may be summarized as follows:

(*a*) When a contralateral subliminal stimulus is applied, the monaural threshold is decreased, whatever the relative frequencies of the two notes used. The reduction is such that if the energies applied to each ear are expressed as fractions of the corresponding monaural threshold energies, the sum of the two fractions is unity (Hughes 1938).

(*b*) When the monaural threshold intensity is determined with, and without, an ipsilateral subliminal stimulus, the intensity required is less in the presence of the additional stimulus, and tends to decrease as the intensity of that stimulus is increased. The decrease in intensity is not as great as in the corresponding binaural case, but appears to be of the same order for all pairs of frequencies applied to the ear for a definite intensity of the subliminal stimulus (§ 1 above).

(*c*) The monaural threshold is unaffected by contralateral audible stimuli for intensities of the latter up to 15 db. above their threshold. This holds for notes differing considerably in pitch, but there is a rise in threshold when the notes are in unison (§ 3 above).

(*d*) No definite variation in the monaural threshold occurred in the presence of audible ipsilateral stimuli as far as could be ascertained under the conditions of the experiments (§ 2 above).

Paragraph (*a*): Summation is thus complete with subliminal contralateral stimuli. It is interesting to note that the results of Gage (1932) agree with those found in these experiments. Averaging his results over all the frequencies he used (notes in unison were employed for each ear), he found that a decrease from monaural threshold of 1·0 db. in one ear corresponded to a decrease of 5·6 db. in the other. Since his steps of attenuation were of 2·5 db., and there is a large "scatter" among his results, these figures agree well with those of the present work giving $(E_1/E_0)_L + (E_1/E_0)_R = 1{\cdot}07$ instead of unity.

Paragraph (*b*): With subliminal stimuli applied to one ear the summation again occurs, but is not complete. If the two inputs concerned in the latter case arrived at the final centre by entirely independent paths there would seem to be no reason for this difference: it may be tentatively suggested that when the two stimuli are on the same side occlusion may occur at some stage before the final centre is reached. The experimental results seem to show that some such effect does take place, since the total energy required at the "binaural threshold" appears to increase with the intensity of the subliminal note, indicating an increasing degree of "overlap" among the units.

Paragraph (*c*): Since no significant threshold change could be found when contralateral audible stimuli were present, it would appear that a different mechanism comes into play when the threshold has been reached in one ear, and the "summation effect" so definitely present in the case of contralateral subliminal stimulation is no longer found. A new psychological factor has been introduced in these experiments; when stimulation was subliminal, no audible sensation was produced until the "threshold" had been reached, but in this case an audible sensation was present throughout its determination, and the attention of the observer was engaged by it before the presence of the note of variable intensity was detected. The results obtained agree with those given by Fletcher (1929) for the binaural masking of one pure tone by another.

The results found when the two notes were in unison suggest that localization, rather than audibility, plays the main part in determining the "threshold" in this case. It appears that the presence of the note of variable intensity cannot be detected unless its intensity is sufficient to produce almost central localization, i.e. to cause the combined note to appear to issue from a source located somewhere near the median plane. If it is much less than this, the location of the note remains wholly in the ear in which the intensity has already been fixed at some audible level, and the change of location due to the sudden application of the note of variable intensity to the other ear can no longer be distinguished.

Paragraph (*d*): The absence of any definite threshold variation with ipsilateral audible stimuli may also be due to occlusion, though the experimental results are not numerous enough to permit any deduction to be made.

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Summary

Application of contralateral subliminal stimuli decreases the monaural threshold, whatever the relative frequencies of the two notes used. Ipsilateral subliminal stimuli also decrease the threshold, but less than do corresponding contralateral stimuli. For two notes differing considerably in pitch the monaural threshold is unaffected by contralateral audible stimuli of intensities up to 15 db. above their threshold. No definite threshold variation occurred when similar audible ipsilateral stimuli were applied.

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The influence of diabetogenic anterior pituitary extracts on the islets of Langerhans in dogs

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[Plates 10–12]

Introduction

It has been shown by Young (1937, 1938) that the daily injection into dogs of large amounts of a crude anterior pituitary extract can induce, in a relatively short time, a clearly defined diabetic condition, which continues after the injections cease and becomes permanently established. These observations have been confirmed by Campbell and Best (1938) and by Dohan and Lukens (1939).

An account of the histological changes in the pancreas and other organs of two dogs made permanently diabetic by treatment with crude anterior pituitary extract in this way, has already been published (Richardson and Young 1938). Nine further animals have been available for examination including several that had been treated with fractionated extracts instead of the original crude saline extract. With this additional material it has been possible to confirm and extend our original observations on the effects of the diabetogenic extract on the islets of Langerhans.

Methods

Since the permanent diabetes induced in these dogs is not necessarily accompanied by gross pathological lesions in the islets of Langerhans, care has been taken to obtain the most suitable fixation for specific staining of the alpha and beta cells. It is proposed to describe the technique used in some detail as, in our experience, the pancreas of the dog is rather more difficult to preserve than human or rodent glands.

Fixation. Helly's fluid (Zenker-formol) was found to be the most successful fixative for the islets of Langerhans provided the formalin was carefully neutralized before adding it to the Zenker's solution. The zymogen granules and specific granules of the islet cells are dissolved or rendered unstainable if acid commercial formalin is used in the fixative or if the formalin is slightly alkaline. The addition of a very small amount of brom-thymol blue powder to the commercial formalin, followed by enough solid $CaCO_3$ or $MgCO_3$, to give a definite yellowish green colour (*p*H 6·8) in the stock solution, has proved satisfactory.

Many small blocks of pancreas, not more than 5 mm. thick were taken from the head, body and tail of the organ for fixation and, after half an hour's immersion in the Zenker-formol, the blocks were sliced again to a thickness of 2 mm. The fixation was continued for 24 hr. Zenker-formol penetrates the pancreas very slowly so that only a limited number of sections from the surface of each block could be used for critical staining. It was necessary to have a large number of blocks to provide an adequate number of sample sections from all parts of the pancreas. The fixative was washed out and the specimens dehydrated in alcohol in the usual manner.

Imbedding and sectioning. The dehydrated blocks were placed in alcohol-ether overnight, and then infiltrated with collodion (Necol), using solutions of graded concentration over a period of several weeks. The final imbedding was carried out in 20–25 % solution which was hardened immediately in chloroform vapour and then in pure chloroform for 24 hr. The solid collodion blocks were cleared in benzol and transferred to a paraffin wax mixture at 37° C overnight and then infiltrated with soft wax at 50° C. The sections were cut with a sliding microtome at 4μ and attached to slides with egg albumen.

Staining. A haematoxylin stain has been found to be suitable for differentiation of the alpha and beta cells of the islets and can be applied to large batches of slides without the necessity for carefully controlled differentiation of each individual slide. After removal of wax and collodion, the sections were passed through iodine-alcohol to water and mordanted for 24 hr. in 4 % ferric alum. After rinsing in water they were stained for 24 hr. in 0·5 % aq. haematoxylin (Grübler). The sections were then differentiated in ferric alum to show the same details as with a routine Heidenhain iron haematoxylin staining, rinsed in water, and transferred to Mallory's phosphotungstic acid-haematoxylin at 37° C for 24 hr. The Mallory haematoxylin was ripened with potassium permanganate. The sections required no further differentiation beyond rinsing off excess haematoxylin with 90 % alcohol, after which they were dehydrated and mounted in Canada balsam.

By this technique the granules of the beta cells were stained pale grey in contrast to the dark blue, coarser granules of the alpha cells. The zymogen granules of the acinar cells were very intensely stained, and some staining of the mitochondria both in the acinar epithelium and in the islets was obtained. There was little difficulty, however, in distinguishing between mitochondria and the specific cytoplasmic granules in the islet cells.

RESULTS

(1) *Pancreatic tissue from dogs in which a permanently diabetic condition had been established*

The following three animals had become diabetic after a short series of daily injections of crude anterior pituitary extract. The diabetic condition continued after the cessation of injections and had apparently become permanent. Although treatment with insulin was not necessary in all cases for the survival of these animals, they had received insulin therapy for short experimental periods before death, in the course of observations on their metabolism.

Dog 44. This permanently diabetic animal was depancreatized 401 days after permanent diabetes had been established. It was given insulin injections for a short period before the operation. The insulin content of the pancreas was very low (2·5 units, compared with a normal value of about 80 units).

The acinar tissue was normal without evidence of pancreatitis or obstruction of the duct system, although the smaller intralobular ducts showed extensive vacuolation of their epithelium. This vacuolation was remarkably specific in its distribution, being confined to the cubical epithelium of the intralobular duct system, including the centro-acinar cells, whereas the columnar epithelium of the larger interlobular ducts remained unchanged. A similar vacuolation has been described by Allen (1922) in dogs made diabetic by partial pancreatectomy and suitable dietary measures but the abnormality of the duct epithelium in his animals was present only in cases of severe diabetes, being absent in the early stages when the islets were still intact.

From examination of frozen sections stained with Sudan III, the vacuolation recorded in our previous report (Richardson and Young 1938) was considered to be due to fatty infiltration. Fat is common in the duct epithelium of all the diabetic dogs but some vacuolation, present in routine sections from the present series, was due to glycogen deposition. The two substances have been demonstrated simultaneously in preparations fixed in Zieglwollner's solution followed by staining with Best's carmine. Some of the vacuolation was due, on the other hand, to neither of these substances, and appeared to be the result of some hydropic change. No similar change has ever been observed in the columnar epithelium lining the larger ducts, even in the cases in which obstruction of these ducts had occurred. The term hydropic degeneration, which is applicable to the changes found in the islet cells, is not so suitable for describing the condition of the duct epithelium. The duct epithelial cells accumulate vacuoles in their cytoplasm, but they do not hypertrophy or degenerate to the extent of leaving spaces devoid of nuclei. Complete destruction of the duct epithelium takes place only in necrotic areas following pancreatitis. Apparently the vacuolation of the intralobular duct epithelium has no immediate effect on the secretory activity of the acinar cells and the duct system continues to function normally for long periods in diabetic animals.

The majority of the islets in the pancreas of dog 44 were reduced to small groups of alpha cells with a few agranular beta cells. Some islets contained no beta cells although occasionally, in other islets, normal beta cells could be distinguished. Without any quantitative measurement of the islet tissue it was obvious that there had been a profound reduction in the number of beta cells, and that the remaining cells of this type were largely in a state of functional exhaustion, or were elaborating cytoplasmic granules at a very reduced rate.

Dog 50. This animal, suffering from a cold, was killed 268 days after permanent diabetes had been established. It had been given a period of experimental insulin therapy which was discontinued 14 days before death.

The entire pancreas was abnormal in this dog. Acute pancreatitis, with obstruction of the large interlobular ducts (figure 4 A, plate 10), had resulted in extensive vacuolation of the acinar epithelium, and distension of the smaller ducts, although the production of zymogen granules was still apparently unaffected. The epithelium of the intralobular ducts was vacuolated and distorted to a maximum degree. Necrotic areas were present in some of the lobules.

A few hyalinized islets could be distinguished (figure 9, plate 12) and occasionally an apparently disordered clump of alpha cells remained, which suggested that a reduction in the amount of islet tissue had occurred some time previously, as in dog 44. No normal beta cells were found in this pancreas but, owing to the abnormal state of the pancreas as a whole, it is impossible to state definitely whether the beta cells had indeed been entirely obliterated. Unfortunately no insulin assay was made on this gland.

Dog 51. Following the sudden withdrawal of insulin which had been given for a short period, this dog lapsed into coma and was killed 177 days after it had become permanently diabetic.

There was slight inflammation of the interlobular connective tissue in this pancreas and some indication that obstruction of the duct system was beginning, although the acinar epithelium was not extensively vacuolated. The intralobular duct epithelium was again infiltrated with fat and showed hydropic changes. The majority of the islets were occupied by hyaline material which had replaced the beta cells, leaving compact groups of alpha cells, generally at the margin of the islet area (figure 10, plate 12). The normal arrangement of the alpha cells appeared in some cases to be undisturbed by the small deposits of hyaline material replacing the beta cells. The replacement of the beta cells in such islets was remarkably specific (figure 11, plate 12) and was probably proceeding at the time of death. Occasionally a group of alpha cells, with a few normal or agranular beta cells, could be detected (figure 12, plate 12) and these were not accompanied by hyaline material, but it is highly probable that functional beta cell tissue in this pancreas was in the last stages of degeneration.

(2) *The pancreas in dogs injected with fractionated anterior pituitary extracts and in which diabetic symptoms either did not appear or were not induced permanently*

Dog 55. This animal was given daily injections of an inactive "albumen" fraction of anterior pituitary extract (Young 1939) and showed no diabetic response. After an interval of one week, the original crude extract was administered resulting in ketonuria but no glycosuria. The dog was killed at a time when the blood sugar level was low.

The acinar tissue showed no change from normal beyond the vacuolation of the intralobular duct epithelium which was present in varying intensity in all animals receiving large doses of anterior pituitary extract. The larger ducts were not obstructed. The majority of the islets examined in this pancreas were small and somewhat irregular in shape suggesting a reduction in cell content. Some of the beta cells were agranular.

Dog 58. After repeated injections of the inactive "euglobulin" fraction of anterior pituitary extract (Young 1939), this dog failed to show diabetic symptoms and was killed.

The acinar tissue of the pancreas was normal, apart from mild vacuolation of the intralobular duct epithelium, which again indicated that the animal had been treated with a pituitary extract, although in this case the extract was ineffective in causing diabetic symptoms. The islets were mostly normal, a few beta cells in some sections showing hydropic degeneration or loss of cytoplasmic granules. No mitotic activity in the islets was observed.

Dog 61. The "albumen" fraction of anterior pituitary extract was given to this dog without inducing any diabetic response. The pancreas in this case was less affected than in dog 58 and was, in fact, almost normal. Slight vacuolation in some of the intralobular ducts was the only variation from the normal to be seen in the exocrine part of the gland. The islets were normal in size, some containing a few beta cells showing complete or partial loss of cytoplasmic granules. The alpha cells were unaffected. The loss of beta cell granules was so slight as to resemble that which might be obtained by small intravenous injections of sugar.

Dog 72. This animal responded to injections of the "globulin" fraction of anterior pituitary extract (Young 1939) by becoming temporarily diabetic, but it subsequently became resistant to increased dosages and was sugar-free at the end of the experiment. At this time the dog was still resistant to the action of insulin.

The intralobular duct epithelium was very slightly vacuolated; otherwise the acinar tissue was normal. The islets were small but composed uniformly of granular alpha and beta cells with no evidence of hydropic degeneration or functional exhaustion. Mitotic figures were present in several small islets.

Dog 74. Following injections of the inactive "albumen" fraction, a crude saline anterior lobe extract was administered to this dog which then became temporarily diabetic. Subsequently it became resistant to the diabetogenic action of the crude extract and was killed when the diabetic symptoms had disappeared.

The vacuolation of the duct epithelium in this pancreas was very slight and was confined to a few cells in individual ducts. The islets appeared to be normal in every respect (figure 5, plate 11).

From one block, taken from the tail of this pancreas, evidence has been obtained of the apparent transformation of individual acini into islet tissue (figure 8, plate 11). In a single large islet examined in serial sections, the remains of two acini were clearly distinguishable; one located at the periphery but not in continuity with the adjacent acinar tissue, and the other situated centrally and entirely surrounded by the rest of the islet tissue. In the peripheral area the form of the original acinus was still distinct and the centro-acinar cell still unaltered. The zymogen granules were present in numbers comparable to the rest of the acinar tissue but the basal cytoplasm of the modified acinar cells was filled with granules staining precisely like the alpha cell granules in the rest of the islet. The more centrally situated acinus was less clearly defined as the zymogen granules had become largely depleted. In this instance the apparent transformation into alpha cells was further advanced.

Dog 75. After injections of crude extract inactivated by heating, treatment with the original crude extract resulted in the development of a mildly diabetic condition in this dog. The blood sugar level was high at the time when the dog was killed. No abnormality could be found in the exocrine part of this animal's pancreas beyond an extensive vacuolation of the duct epithelium. Hydropic degeneration in individual beta cells was very common in the islets. The examples of stages in this degenerative process, illustrated in figures 1–3, plate 10, have been taken from this pancreas.

There is no longer any doubt that hydropic degeneration is a clearly characterized change occurring in islet cells, although it is not invariably present in the pancreas of diabetic dogs and is encountered only rarely in the human pancreas. Hydropic degeneration is not an artefact due to post-mortem autolysis, as some authors have claimed, and should not be confused with cytoplasmic shrinkage and vacuolation such as Warren (1938) has shown to occur 4 hr. post mortem in the human pancreas. The accumulation of small vacuoles in the cytoplasm of an islet cell appears to be the first stage in the process. The cytoplasmic granules may have been depleted previously or pale staining granules may be present throughout the degenerating cell. When the vacuoles become distributed throughout the entire cytoplasm, the cell hypertrophies and its nucleus undergoes pycnosis. Some of the vacuoles coalesce together and finally, with the disappearance of the nucleus, a large space is produced in the islet which is bridged over in some cases by a fine network of residual cytoplasm. The degeneration generally occurs in scattered individual beta cells under the conditions of the present experiments. It does not affect large groups of beta cells in any one islet.

Mitotic activity was also observed in some islets of the pancreas of dog 75, figure 3, plate 10. The remainder of the islet tissue showed marked loss of staining reaction of the beta cell granules, some beta cells being entirely agranular, figure 3, plate 10, figure 6, plate 11.

It is proposed to distinguish in this case between (*a*) loss of granules in the cytoplasm of the beta cells leading to the agranular cell, the cytoplasm of which almost entirely fails to take up stain and is, in this respect, comparable to the cytoplasm of the intralobular duct epithelium, and (*b*) loss of staining affinity of the cytoplasmic granules. In the latter case the granules are present and often completely fill the cytoplasm but they stain so faintly that under normal microscopical conditions they are almost invisible. By using a green filter, however, the granules can be more clearly distinguished and can be recorded in a photomicrograph. This distinction, which may not be important physiologically, is apparently not due to variations in technique. In the present series, the block of pancreas from dogs 74 and 75 was fixed in the same solution of Zenker-formol and the sections were stained together in the same haematoxylin solution. In the case of dog 74, the beta cell granules were consistently stained the same pale grey colour and were easily distinguishable with ordinary illumination, figure 5, plate 11. The granules contribute clearly to the much darker staining of the islet as a whole, whereas in the pancreas of dog 75, the islets were very pale staining, figure 6, plate 11, and the granules were not recorded by photography except under suitable screening conditions and over-exposure in printing.

Discussion

The results of the histological examination of the islets of Langerhans in fourteen dogs are summarized in table 1. It is fully realized that this series of animals is not sufficiently large for reliable comparisons to be made at present between the pathology of human diabetes and experimental diabetes induced by anterior pituitary extracts. It is important, however, to distinguish between the immediate histological effects of injections of anterior pituitary extracts on the pancreas and the effects found after a prolonged period of permanent diabetes. It is also important to attempt to correlate the histological state of the islet tissue as far as possible with the severity of the diabetic symptoms or with the failure of these symptoms to appear. The following four distinct histological changes in the pancreas will now be discussed with reference to these considerations.

(1) *The vacuolation of the intralobular duct epithelium*

In the previous report on the histological changes in the pancreas in diabetic dogs (Richardson and Young 1938), the vacuolation of the intralobular duct epithelium was mentioned as occurring markedly in the two permanently diabetic dogs 28 and 40. In the present series this process was also more prominent in the animals which had been made diabetic by treatment with a crude saline extract but, even with the fractionated extracts, which produced no obvious diabetes, some degree of vacuolation was always found. The vacuolation of the intralobular duct epithelium was the most consistent change in the pancreas induced by large doses of anterior pituitary extracts.

The significance of this phenomenon cannot be explained at present, but it should be stressed that it occurs in all dogs treated with the various

TABLE I

No. of dog	Pituitary extract used	Diabetic state	Period of permanent diabetes	Condition of dog at time pancreas was taken	Condition of islets: Hydropic degeneration	Condition of islets: Degranulation of beta cells	Condition of islets: Hyalinization	Remarks
15	Crude	Temporary	...	Refractory	+ +	+	–	Richardson and Young (1938)
16	Crude	Temporary	...	Diabetic	+ +	+	–	Richardson and Young (1938)
28	Crude	Permanent	44 weeks	Coma following insulin withdrawal	–	–	+ + +	Richardson and Young (1938)
40	Crude	Permanent	30 weeks	Biopsy	...	+ +	–	Richardson and Young (1938) (later de-pancreatized)
41	Crude	Temporary	...	Refractory one month after last injection	–	–	–	Richardson and Young (1938)
44	Crude	Permanent	58 weeks	Severe diabetes	–	+ + + +	–	Islets reduced to groups of alpha cells
50	Crude	Permanent	38 weeks	Insulin withdrawn 14 days before death	–	–	+ + +	...
51	Crude	Permanent	25 weeks	Coma following insulin withdrawal	–	–	+ +	...
55	"Albumen" then crude	Ketonuria but no glycosuria	...	Refractory	–	+	–	...
58	"Euglobulin"	...	...	Normal	In few beta cells	–	–	...
61	"Albumen"	...	...	Normal	–	Few partly degranulated	–	...
72	"Globulin"	Temporary	...	Refractory	–	–	–	Small normal islets
74	"Albumen" then crude	Temporary	...	Refractory	–	–	–	...
75	Heat inactivated, then crude	Temporary	...	Mildly diabetic	+ +	+ +	–	...

extracts referred to in this series of experiments. The obstruction of the duct system found in dogs 28 and 50 occurred actually in the interlobular branches, and did not appear to be directly related to the condition of the intralobular ducts. In a later part of this discussion the changes in the duct epithelium will be referred to in connexion with the problem of the regeneration of islet tissue in the pancreas of the diabetic animals.

(2) *Hydropic degeneration in islet cells*

Hydropic degeneration in beta cells has occurred most consistently in the islets of those dogs which were killed early in the course of the experiments at a time when the blood sugar level was high. The degeneration was more common in the material examined during the period of injections and was not a prominent feature of the permanently diabetic animals. In the permanently diabetic dog 40, hydropic degeneration was found only in a few beta cells in all the blocks of pancreas which have been examined both from the biopsy specimen and from the rest of the gland taken later when the animal was de-pancreatized, figure 7, plate 11.

It has been assumed that hydropic degeneration in beta cells is due to functional strain in response to diabetic conditions. In the well-known experiments of Allen (1922), hydropic degeneration involved the beta cell tissue as a whole, in the small portion of intact pancreas remaining, in dogs in which partial pancreatectomy and suitable dietary measures had induced symptoms of diabetes. Woerner (1938) has found the lesion in the islets of guinea-pigs following prolonged intravenous injection of dextrose. It is important, therefore, to consider what part hydropic degeneration plays in the present experiments.

As is well known, the normal pancreas contains an amount of islet tissue which is greatly in excess of that required to supply the normal demands of the body for insulin. In the dog, the experiments of Allen (1922) have indicated that the pancreas may be reduced by partial pancreatectomy to as little as one-ninth of its original size without diabetic symptoms occurring. The question now arises: can the diabetic symptoms induced by anterior pituitary extract be the result of a reduction of the total amount of beta cell tissue in the islets by hydropic degeneration, so that the amount of this tissue remaining is actually less than that present in a small fraction of the normal gland? This is certainly not the case in dogs 16 and 40. It is probable that hydropic degeneration is the result of a period of excessive strain on the islet cells occurring particularly during the period of injections with pituitary extract. When the injections are discontinued and the strain is reduced, the rate of the degeneration becomes slower or it may even cease. We have no means of following the sequence of events in the permanently diabetic dogs, except to record that cells undergoing hydropic degeneration are rare, but nevertheless it may be reasonably assumed, without quantitative measurements of the amount of islet tissue, that a considerable reduction in islet tissue has occurred in some instances during the period of permanent diabetes. Degeneration of individual beta cells, taking place even slowly over a period of months, would account for such a reduction. It would lead eventually to small groups of alpha cells as the last remains of the islet tissue. Such is the condition of the islets in dog 50 and in some of the dogs in Allen's experiments.

(3) *Degranulation of the islet cells*

The most common change in the islets, following injections of active anterior pituitary extracts, is the loss of some or all of the cytoplasmic granules in the beta cells. It has already been recorded that this loss of cytoplasmic granules may be apparently the only significant change in the islets during many months of permanent diabetes (dog 40, Richardson and Young 1938).

In dog 75 there is evidence that the beta cell granules may respond to a high blood sugar level merely by losing their normal affinity for stains, in which case suitable light filters are necessary to reveal the granules. Although this fine distinction in relative staining intensities does not appear to be due to technical variations, some caution must always be taken in using minute cytological changes as evidence of an alteration in physiological function. Houssay and Foglia (1936) have observed that the pancreas of a dog exhibiting temporary diabetic symptoms as the result of injections of anterior pituitary extract secretes less insulin than usual. If the loss of staining affinity of the beta cell granules was known to occur in glands in which the rate of secretion of insulin was very low, more emphasis might be placed on this phenomenon.

The evidence obtained from the reduced granule content of the beta cells indicates that, at some time or other during the experiment, a cell has partly or completely lost its cytoplasmic granules, possibly through functional exhaustion. We do not know, however, whether the secretory activity of the degranulated beta cells is temporarily depressed or is actually increased. In the present material it is impossible to correlate closely the extent of depletion of beta cell granules, or their loss of affinity for stain, with the physiological condition of the animal. Inactive anterior pituitary extracts, which have failed entirely to induce even temporary diabetic symptoms, may cause partial loss of granules in some of the islets. Nevertheless, extensive depletion has been found only in those animals with high blood sugar levels or in a permanently diabetic state. In dog 40, as has already been mentioned, the loss of cytoplasmic granules in beta cells was apparently the only significant change but in dog 44 there had been a considerable reduction in beta cell tissue so that the majority of the islets consisted of groups of alpha cells. It is interesting to note that the severity of the diabetic symptoms in dog 44 was much greater than in dog 40. Although we may not expect to find more than an extensive loss of beta cell granules in any diabetic animal, the more severe cases appear to be associated with a great reduction in the amount of granular beta cells or a replacement of the islets by hyaline material. The latter point will be discussed in the next section.

Woerner (1938) has described a loss of cytoplasmic granules in the islets of guinea-pigs following intravenous injections of dextrose. He also found degenerative changes in both alpha and beta cells as well as in the intralobular duct and acinar epithelia, following initial periods of exhaustion and hyperplasia of the islet tissue. His experiments have demonstrated that such a considerable series of changes in islet tissue do not necessarily result in a diabetic condition. It is quite possible, however, as he suggests, that from the quantitative point of view the amount of degeneration and degranulation in the islet tissue had been insufficient to overcome the large reserve of islet tissue in the intact pancreas.

(4) *Hyalinization of the islets*

The replacement of islets by hyaline material has been observed only in dogs in which the pancreas was examined at least five months after the diabetic condition had been established. Dog 28 (Richardson and Young 1938) and dog 50 in the present series both showed apparently complete hyalinization of islet tissue. In dog 51 the majority of the islets had become hyalinized to the extent of replacing nearly all the beta cells but leaving groups of alpha cells still intact. This dog had not been diabetic for as long a period as the other two. Hyalinization of islet tissue does not occur, however, in all cases of permanent diabetes induced by anterior pituitary extract, nor is this type of lesion necessarily associated with severe diabetic symptoms or with the length of time during which the animal exhibits these symptoms. Both the dogs 40 and 44 had been diabetic for longer periods than dogs 28, 50 and 51 and yet no hyalinization had occurred.

It is possibly significant that the three dogs with hyalinized islets had all received a short period of insulin therapy which had been abruptly discontinued a short time before death. Two of the dogs, 28 and 51, developed coma soon after the withdrawal of insulin. Acute pancreatitis and obstruction of the major ducts was found in dogs 28 and 50, but was absent in dog 51.

(5) *Regeneration of islet tissue*

In the present experiments, the possible regeneration of islet tissue in the adult dog pancreas is of interest in connexion with the resistance shown by some of the animals to the diabetogenic action of crude extracts. It has been concluded from the statistical examination of frequency distribution of volume of the islands of Langerhans in the pancreas of man, monkeys and dogs (Thompson, Tennant and Hussey 1933) that new islets are probably not formed after a prenatal stage in life. Under experimental conditions, however, an increase of islet tissue in adult animals has been recorded by many authors, particularly in rodents.

Cell division in existing islet tissue has been found to occur in two dogs of the previous series (Richardson and Young 1938) and in dogs 72 and 75 of the present series. The pancreas in these animals was examined immediately after injections of anterior pituitary extract had been discontinued. In each case the mitotic activity in beta cells was accompanied by hydropic degeneration in adjacent beta cells. This hyperplasia may be simply a compensatory mechanism for replacing beta cells lost by degeneration.

Woerner (1938) has found an extensive increase in islet tissue in his animals which followed for a short period after an initial phase of exhaustion of the beta granules. In the dog it is possible that hyperplasia of islet tissue accounts for the production of sufficient beta cells to overcome the diabetogenic action of increased doses of anterior pituitary extract, thus explaining the resistance found in dogs 15, 41, 55 and 74. The experiments of Woerner (1938) indicate that, in guinea-pigs at least, the period is very limited during which hyperplasia, of islet tissue undergoing functional strain, is possible, and occurs quite early in the course of the experiments. It is interesting to note that the hyperplastic phase in guinea-pigs followed immediately after the degranulation of the beta cells. The hydropic degeneration actually took place after proliferation in the islets in Woerner's experiments. The increase in islet tissue was therefore not simply a compensation for destruction of some of the beta cells.

Evidence for the existence of a "pancreotropic" factor in anterior pituitary extracts, suggested by Anselmino, Herold and Hoffmann (1933), has been obtained by the quantitative estimation of islet tissue in rats injected with the same crude saline extract of the anterior lobe as was used in some of the present dogs (Richardson and Young 1937). This factor may account for the hyperplasia of islet tissue in the dog and is probably released from the anterior lobe of the pituitary in intact guinea-pigs undergoing intravenous injections of dextrose.

The newly formed islet tissue in Woerner's experiments was derived from three sources, viz. mitoses in pre-existing beta cells, by transformation of duct and centro-acinar cells into beta cells and by direct transformation of acinar cells into beta cells. By demonstrating the existence of a transitional cell-type between acinar and beta cells, which contains both zymogen and specific islet cell granules in its cytoplasm, he has presumably settled the long-standing controversy concerning the possibility of differentiated acinar epithelium being transformed into islet cells. Woerner was able to check the histological evidence of hyperplasia of islet tissue by the examination of whole mounts of pancreas in which the entire islet tissue had been stained selectively with janus green. The present material does not show conclusive evidence of the transformation of acinar epithelium into islet cells, except in the one instance which has been described in the pancreas of dog 74.

If the intralobular duct epithelium is active in the formation of new islet tissue in dogs, it is important to note the early damage which is caused in this epithelium by the anterior pituitary extracts. The vacuolation in the duct epithelium persists over long periods and extends completely throughout all the lobules in the dogs that have become permanently diabetic. This probably renders the duct epithelium ineffective as a source of new islet tissue from the commencement of the experiments.

CONCLUSIONS

It may be concluded tentatively, from the present material, that the injections of crude anterior pituitary extract and the "globulin" fraction of this extract, cause initially both degranulation and hydropic degeneration in the beta cells of the islets of Langerhans. At least a temporary phase of diabetes is associated with these changes. Partial degranulation and hydropic degeneration in beta cells can apparently be induced by anterior pituitary extracts which do not cause a demonstrable diabetes. But it is probable that these changes must be quantitatively significant in the islet tissue as a whole before diabetic symptoms occur. Hydropic degeneration alone cannot account for a reduction in islet tissue to an amount which is less than that required for the maintenance of a non-diabetic condition.

In the production of a permanently diabetic condition, the first change in the beta cells during the period of injections of pituitary extract is presumably beta cell degranulation and associated with this change is a loss of beta cells by hydropic degeneration. Thus when the permanent diabetes is established and the injections are discontinued, the beta cells which remain in the islets are presumably depleted of cytoplasmic granules to a large extent. Subsequently hydropic degeneration may continue at a slow rate to reduce the number of beta cells in each islet, or the islets may be hyalinized before such a reduction takes place.

The development of refractoriness to the diabetogenic action of crude extract is possibly associated with islet hyperplasia at the time of injection, but a quantitative estimation of the total amount of islet tissue, combined with an insulin assay of the same pancreas, would probably provide more reliable evidence for this hypothesis than the cytological examination of even large numbers of individual islets.

The photomicrographs are the work of Mr F. J. Pittock, F.R.P.S., to whom the author wishes to express his thanks.

SUMMARY

1. The early histological changes in the pancreas of dogs following the administration of large amounts of a diabetogenic anterior pituitary extract are as follows:

(*a*) partial or complete degranulation of beta cells in the islets of Langerhans,

(*b*) hydropic degeneration in individual beta cells,

(*c*) mitotic activity in some islets,

(*d*) vacuolation of the intralobular duct epithelium.

2. The vacuolation of the duct epithelium and slight loss of cytoplasmic granules in beta cells followed the injection of fractionated anterior pituitary extracts which failed to produce diabetic symptoms.

3. In dogs in which the diabetic condition had been established, for prolonged periods following the cessation of injections, the islets have shown one of the three following conditions:

(*a*) degranulation of the beta cells,

(*b*) a reduction of islet tissue to groups of alpha cells with a few agranular or normal beta cells,

(*c*) hyalinization of the islets which had apparently suppressed the islet tissue as a whole or had replaced the beta cells selectively.

4. Hyalinization of the islets does not appear to be related to the length

of time during which the diabetic symptoms had continued. It has been observed only in those animals whose islets had been subjected to the strain of a sudden withdrawal of insulin therapy following a short experimental period.

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Description of Plates

All the figures are from untouched photomicrographs of sections stained with the special haematoxylin combination described in the text.

Plate 10

Figure 1. Dog 75, an islet showing various stages in the process of hydropic degeneration in beta cells. (The print has been over-exposed to show the faintly stained cytoplasmic granules in these cells.) Mag. × 650.

Figure 2. Dog 75, an islet showing further examples of hydropic degeneration in beta cells. Mag. × 600.

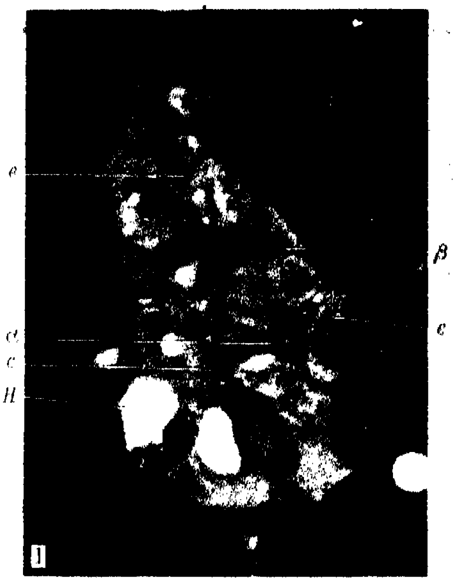
Figure 3. Dog 75, showing hydropic degeneration in two beta cells, and mitosis in a third. The remaining beta cells show depletion and pale staining of their cytoplasmic granules. Mag. × 465.

Figure 4. A. Dog 50, an interlobular duct showing obstruction of its lumen. The columnar epithelium is apparently normal and should be compared with the intralobular duct epithelium from another dog shown below in figure 4B. Mag. × 250. B. Dog 55, a mild degree of vacuolation in the epithelium of an intralobular duct. Mag. × 350.

Plate 11

Figure 5. Dog 74, a normal islet in which the alpha and beta cells are filled with granules which contribute to the general grey tone of the islet as a whole. Mag. × 300.

Figure 6. Dog 75, an islet in which the majority of the beta cells are partly degranulated. Compare the pale staining of the remaining granules with that in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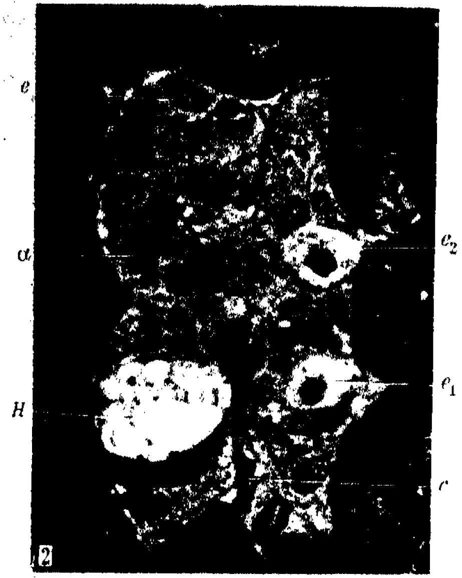
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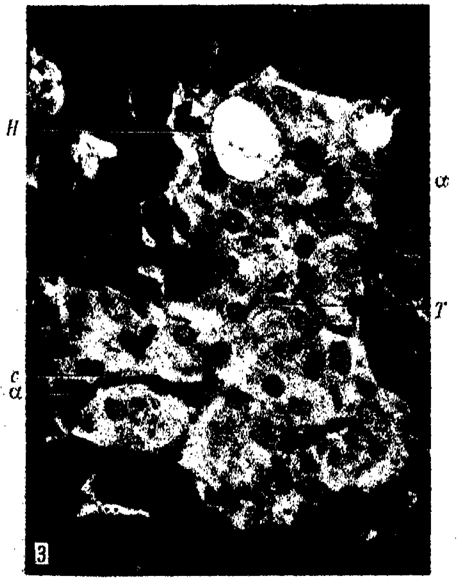
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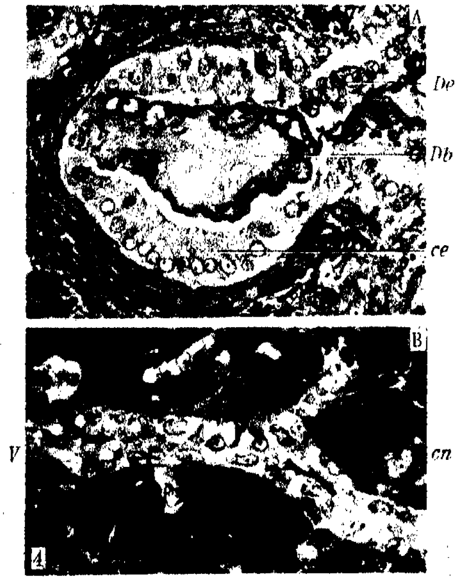
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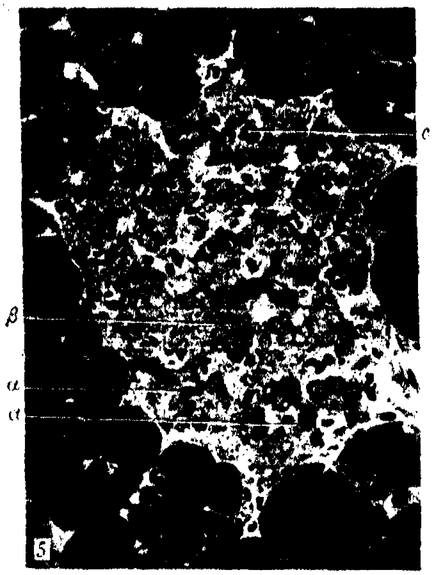
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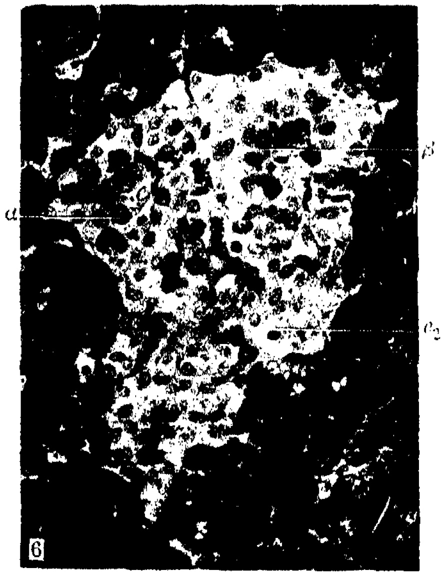
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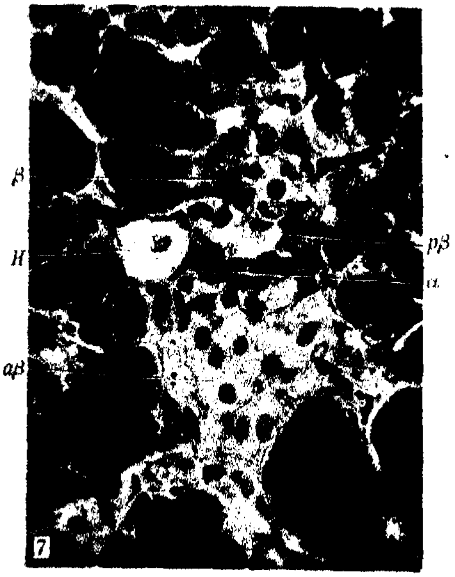
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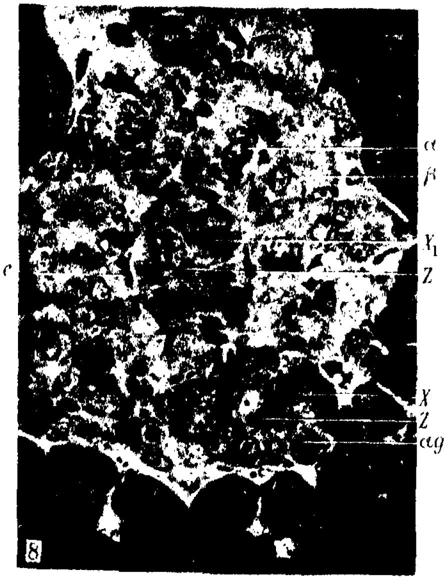
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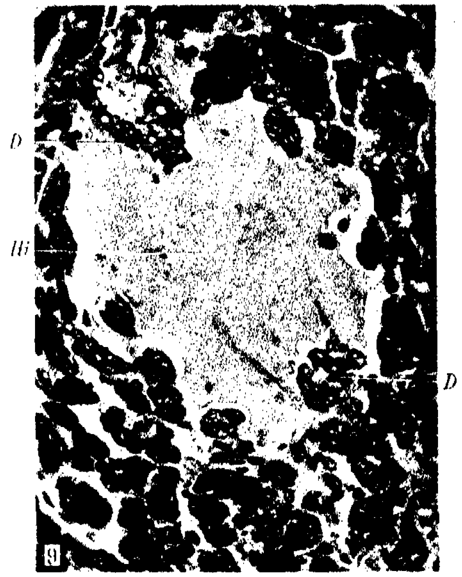
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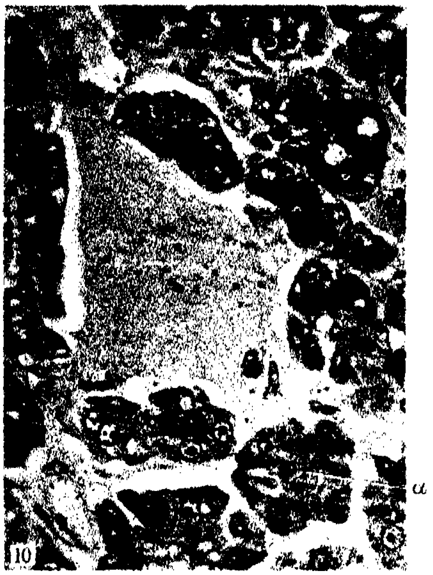
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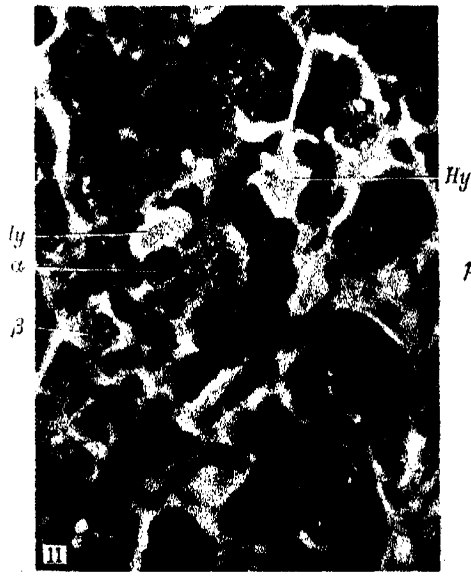
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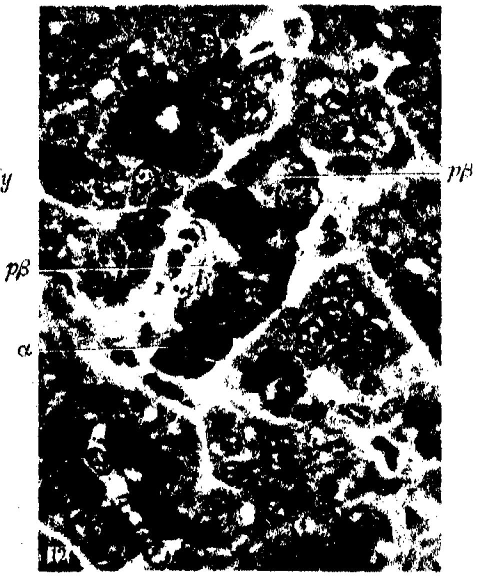
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figure 5. Also compare with the staining intensity of the lower half of the islet in figure 7, which is almost completely agranular. Mag. × 275.

FIGURE 7. Dog 40 (see Richardson and Young 1938), an islet from a permanently diabetic animal. The upper half of the islet consists of normal and partly degranulated beta cells; the lower half is almost completely agranular. Mag. × 400.

FIGURE 8. Dog 74, showing the apparent transformation of acini into islet tissue. The acinus "X" contains both zymogen and alpha cell granules. The less distinctly outlined acinus "X_1" in the centre of the islet shows a later stage in the transformation into alpha cells. Mag. × 500.

Plate 12

FIGURE 9. Dog 50, a completely hyalinized islet. The two "stumps" of degenerated duct epithelium were probably in continuity with the original islet tissue. Mag. × 275.

FIGURE 10. Dog 51, a partly hyalinized islet in which a group of pale staining alpha cells is still intact. Mag. × 500.

FIGURE 11. Dog 51, an early stage in the hyalinization of an islet. The homogeneous hyaline material has replaced the majority of the beta cells leaving the alpha cells undisturbed. Mag. × 550.

FIGURE 12. Dog 51, a small islet consisting mainly of alpha cells and a few degranulated beta cells. The condition of this islet is typical of the majority of the islets in dog 44 also. Mag. × 550.

LETTERING IN FIGURES

α = alpha cell.
αg = alpha cell granules.
$a\beta$ = agranular beta cell.
β = beta cell.
c = capillary.
ce = normal columnar epithelium.
cn = centro-acinar cell.
D = remains of intralobular duct (to which islet was probably attached).
Db = debris obstructing lumen.
De = desquamated epithelial cells.
e = early vacuolation phase of hydropic degeneration in beta cell.
e_1 = hydropic degeneration: vacuoles filling half the cytoplasm.
e_2 = hydropic degeneration: vacuoles filling cytoplasm, nucleus pycnotic.
H = last phase of hydropic degeneration.
Hi = hyalinized islet.
Hy = hyaline material replacing beta cells.
$p\beta$ = partly degranulated beta cell.
T = mitotic telophase in beta cell.
V = vacuoles in cubical epithelium.
X = transformed peripheral acinus.
X_1 = centrally placed acinus in which zymogen is disappearing and alpha cell granules are distributed throughout the cytoplasm.
Z = zymogen granules.

The suppression of meiosis and the origin of diplochromosomes

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(*Communicated by Sir Daniel Hall, F.R.S.—Received* 11 *August* 1939)

[Plate 13]

1. Introduction

The effects of abnormal temperatures on the meiotic divisions in various plants have been investigated by Sax (1937), Straub (1938), Matsuura (1937) and others. The effects which they have described arise in two ways: (1) by prevention of the pachytene pairing and subsequent chiasma formation in the chromosomes, and (2) by inhibition of the action of the spindle. In the first case we have a development very similar to that described in numerous hybrids, which can be adequately understood in terms of the irregular behaviour of univalents. They may, for instance, give rise to restitution nuclei at the end of the first division, and divide equationally at the second to give diploid pollen grains (Sax on *Rhoeo* 1936, and *Tradescantia* 1937). The suppression of the spindle gives rise to similar effects in the pollen grains. Separation of the bivalents and wall formation are affected so that diploid and tetraploid grains are formed as well as many unbalanced types.

The present paper gives an account of a new type of derangement produced by high temperatures. In addition to the upset of developmental processes external to the chromosomes, as in the above cases, the internal development of the chromosomes is affected, so that a nucleus at early diplotene changes directly into a pollen-grain resting nucleus.

2. Material and methods

Potted plants of *Fritillaria Meleagris* were placed in incubators for various times. A range of temperature from 30–40° C was used, and the time of treatment varied from 5 hr. to two days. Fixations were made immediately after treatment and at various times following, to obtain a good series of stages up to the first pollen-grain mitosis. 2BE-Gentian violet, acetocarmine and Feulgen smears were used. The Feulgen technique is particularly useful when dealing with small amounts of material; the acid hydrolysis softens the cell-walls so that the cells stick to the slide better. For help in making some of the preparations I am indebted to Mr La Cour. Details of the methods used will be found in La Cour's paper of 1937.

3. NORMAL CHROMOSOME BEHAVIOUR IN *FRITILLARIA MELEAGRIS*

The chromosomes of *Fritillaria Meleagris* have been described by Newton and Darlington (1930). The haploid number is twelve, the complement consisting of ten chromosomes with a subterminal centromere, and two with an approximately median centromere, one arm being slightly longer than the other in both chromosomes. There is a well-defined nucleolar constriction in the long arm of one of the subterminal chromosomes.

At meiosis twelve bivalents are formed, held together by one to five chiasmata, which are strongly proximally localized. Chiasmata very rarely form in the distal regions of the long arms (figure 5, plate 13). The chromatids are jointly coiled in pairs with both major and minor spirals.

4. THE EFFECT OF HEAT ON THE MEIOTIC DIVISIONS

The plants were treated just after pachytene in the anthers. Short period treatments, for instance 5–6 hr. at 30° C or 10 hr. at 38° C during early diplotene have little if any effect on the succeeding meiotic divisions. Tetrad formation is perfectly normal. If, however, the high temperatures are continued through the period of development of the meiotic spindles, numerous abnormalities appear. The following description applies to plants heated for two days at 30° C, the period of treatment covering late pachytene to tetrad formation.

It is important to realize that the type of development may be different in the anthers of the same flower. For instance, in one flower meiosis was suppressed in two anthers whilst in the others the pollen mother cells either managed to get through the first division and formed binucleate pollen grains or formed numerous micronuclei and gradually degenerated. In the untreated flower the anthers may not all be at the same stage of development—a fact which explains the variability after heat treatment, for as we shall see, the effectiveness of the treatment depends on the developmental stage at which the treatment is begun.

Fixations immediately after the treatment show some cells at first metaphase with usually twelve bivalents. There are occasional cells with univalents, but the proportion of unpaired chromosomes is not very much higher than in untreated plants. In thirty pollen mother cells of the heated plant five pairs of univalents were counted, whilst in thirty from an untreated control plant one pair was observed. Further, the heat treatment has no effect on the distribution of the chiasmata within the bivalents. The proximal localization is still maintained. Heating after the pachytene stage has therefore very little or no effect on chiasma formation. Pairing and chiasma formation are complete after pachytene.

Usually, if the cells come to first metaphase, the anaphase follows perfectly normally. But in others, although some of the bivalents separate, others fail to do so or are delayed. There are two reasons for this. First, bivalent orientation is not so regular in the heated plants. At full metaphase, one or two bivalents with normal chiasma configurations may still be lying off the spindle and only orientate after the others have separated. In the second case, although orientation is perfectly normal, the chromatids in certain of the chromosomes fail to unravel at anaphase (see figure 6, plate 13). The situation is like that found by Klingstedt (1939) in grasshopper hybrids. The time correlation between the various processes responsible for the anaphase separation has been upset. The centromere-spindle mechanism appears to have started operating before the complete lapse of chromatid attraction, resulting in stretching and in extreme cases probably breaking the chromosomes. The lagging chromosomes may become incorporated in the telophase nuclei but usually form micronuclei.

In other cells the whole anaphase may fail and the chromosomes may round off to form several micronuclei of a very solid texture. These cells, it appears, gradually degenerate and die. They never form a wall after the heating is discontinued. A similar case was described by Darlington and Thomas (1937) in a hybrid grass. An organized nucleus is apparently necessary for wall-formation to occur.

In the more normal cells with a typical first anaphase, the second division may be completely suppressed. Usually the wall between the two nuclei fails to form. If the plants are returned to normal temperature, these binucleate pollen mother cells lay down a typical pollen-grain wall, the two nuclei lying free in the cytoplasm of a single cell. The photo (figure 8, plate 13) was made after two days at room temperature. Such cells will contain two diploid nuclei, but their further development has not been followed.

Rarely, the interphase pollen mother cell divides again. The two sets of chromosomes may then orientate on one spindle. We have, in effect, the formation of a restitution nucleus. This may be followed by a normal anaphase. If the cell wall forms, this type of development will give diploid grains with normal chromosomes. If the wall formation is again suppressed, a tetraploid grain with normal chromosomes will be formed.

The most extreme alteration in normal events gives a single uninucleate grain as final product. The diplotene nucleus changes directly into a typical pollen-grain resting nucleus (see figure 9, plate 13). The meiotic spiralization cycles and nuclear divisions have been completely suppressed. The photographs are from preparations made two days after the plants were returned to normal temperatures. Pollen-grain wall formation is perfectly normal. The cells become vacuolated, so that the nucleus is often pushed out of the centre to one side, as in the development of normal pollen grains. Sometimes thin transverse septa develop which may cut off small lens-shaped cells or simply project as a ledge into the cavity of the pollen grain. Thus cell division can occur without nuclear division. Katayama (1935) made similar observations in the pollen mother cells of *Triticum* haploids, where small enucleate cells are sometimes cut off following the formation of a restitution nucleus. Presumably a spindle is formed between the nucleus and the surface of the cell and is responsible for laying down the transverse walls.

5. THE POLLEN-GRAIN DIVISION

There are thus three main types of abnormal development possible, the effects of which will be visible in the pollen grain:

(1) Anaphases incomplete and followed by formation of a restitution nucleus—*diploid or tetraploid pollen grain with normal chromosomes.*

(2) First anaphase normal, second division suppressed—diploid nucleus.

(3) Both meiotic divisions suppressed—tetraploid nucleus.

The formation of a restitution nucleus is of common occurrence after failure of pairing of chromosomes at meiosis. This failure of pairing may be consequent on hybridity (e.g. Karpechenko on *Raphano-Brassica*, etc.) or be induced by external conditions such as temperature variation (Sax 1937, on *Tradescantia*, Barber, unpub., on *Uvularia*). That restitution can occur after normal bivalent formation has not previously been observed. As we have seen above, this sequence of events gives diploid or tetraploid pollen grains according as a single or a double restitution has taken place. The chromosomes are normal. Occasionally unbalanced types of grain can arise in this way, as chromosomes are sometimes lost through forming micronuclei during the restitutions. For example, one grain showed only forty-six chromosomes (i.e. $4x-2$).

The formation of restitution nuclei is uncommon and such cells occur scattered singly in anthers in which one or both meiotic divisions have been suppressed. For instance, in an anther in which over 90% of the pollen grains were uninucleate and derived by suppression of both meiotic divisions, four cells were obtained with approximately the tetraploid number of normal chromosomes. A small proportion of the cells also degenerated after forming micronuclei. The formation of a restitution nucleus after complete pairing depends on a delicate adjustment of external conditions.

The complete suppression of a meiotic division gives, of course, the same end result as the formation of a restitution nucleus, i.e. a diploid or tetraploid pollen grain, according as one or both divisions are suppressed. The pollen-grain chromatid divisions take place normally in the resting stage. The centromere, however, requires special conditions for its division. In the normal cell it divides only under the action of the spindle forces. The development of this type of cell will thus be different from that derived from restitution nuclei.

6. THE STRUCTURE OF DIPLOBIVALENTS

Pollen-grain metaphases of cells in which both meiotic divisions were completely suppressed are shown in figures 10, 11, plate 13. They look strikingly like first meiotic divisions with twelve bivalents, except for the greater size of the cell. Each of the bivalents, however, consists of eight chromatids instead of the four of a meiotic bivalent. They are bivalents made up of two diplochromosomes (White 1935). I shall refer to them as "*diplobivalents*". Each chromatid has the mitotic type of spiralization without the major spirals, since one of the diplobivalents often shows the nucleolar constriction (figure 1*a*) which is, of course, not visible at meiosis except in cells without the major spiral (Darlington 1936). The chromatids are not jointly coiled. The bivalents are still held together by "chiasmata" at each of which two pairs of chromatids cross over. They are double or "*diplochiasmata*", and are proximally localized, just as in normal bivalents. The diplobivalents have, therefore, a structure exactly like meiotic bivalents except that two chromatids replace one and the chromatids are not coiled into a joint major spiral. Examples of the diplobivalents are drawn in figure 1. *a* and *b* are from an early metaphase cell in which the chromatid structure is not so distinct as in the others, which are from late metaphase-early anaphase cells. In figure 1c the St Andrew's cross of the original chiasma has opened out to form a St George's cross. Such untwisting is of common occurrence in normal bivalents with a single chiasma.

The frequency of diplochiasmata is probably about the same as the chiasma frequency at the first division of meiosis. A complete analysis in terms of diplochiasmata is difficult, but out of twenty-four cells only one pair of double univalents (*M* chromosomes) was observed (see table 1). At late metaphase there is often a loosening of diplochiasmata in the short arms, so that most of them appear unpaired.

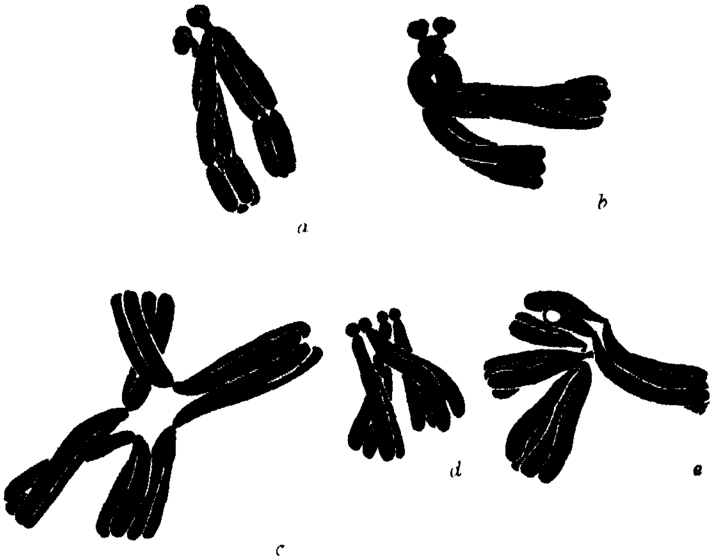

FIGURE 1. Diplobivalents from pollen-grain divisions of *Fritillaria Meleagris*. *a* and *b* are from an early metaphase. *c*, *d* and *e* are from late metaphase or early anaphase cells. × 1800.

TABLE 1

Treatment	No. of cells	No. of univalents	Pairs of univalents per cell
Meiosis, control	30	1	0·03
Meiosis, heated	30	5	0·17
Pollen grain, diplobivalents	24	1	0·04

The complete retention of chiasmata in the same position through a resting stage of about a week (the pollen-grain divisions took place six to seven days after the treatment) shows in a very striking way that there is no movement of the chromosomes relative to one another during a mitotic resting stage, so long at least as the nucleus remains the same size.

At full metaphase very little relational coiling remains in the chromatids. Usually the four are arranged symmetrically in the distal parts, each chromatid forming the corner of a square in cross-section. There is no strict attraction in pairs.

Observations during prophase in diplobivalent nuclei are usually difficult. In a well-fixed nucleus each strand can be seen to be quadruple. Figure 2 represents a complete prophase diplobivalent which by an accident of smearing was pressed free from the others in the cell. At one of the diplochiasmata the pairs of chromatids which cross over are easily visible. The centromere is probably in the end loop, where the four chromatids in each half-bivalent come together. Thus each half-bivalent has the structure of a diplochromosome (White 1935).

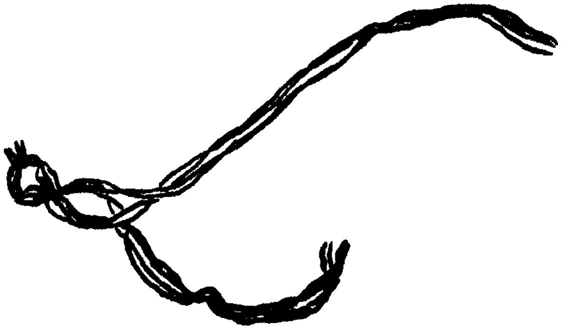

FIGURE 2. A prophase diplobivalent with three diplochiasmata, one in the short arm and two in the long. × 1800.

The centromere is still single at early metaphase (see figure 1 *a* and *b*, and plate 13, figure 10) as is shown by the fact that the four chromatids of each half-bivalent come together at this point. Each orientates separately on the spindle, so that usually the centromere loop is transverse to the spindle and not along it as at meiosis. Thus co-orientation of two centromeres at meiosis must depend on the internal structure of the centromere, not on the presence of chiasmata.

Sometimes the centromeres of a diplobivalent fail to come into the same plane and remain one above the other in polar view. They are evidently not co-orientated, for the proximal portions of the chromosomes are seen not to be under tension. Mechanical difficulties probably prevent the separate orientation of the two centromeres in the same plane.

Another peculiarity in orientation of the diplobivalents is the formation of a "hollow spindle" (see figure 11, plate 13). The bivalents lie round the periphery of the spindle in a few of the cells. At both meiosis and mitosis the chromosomes of *Fritillaria Meleagris* are spaced evenly on the plate. Darlington (1936) has described some hollow spindles in the pollen grains of a triploid *Fritillaria* (*F. pudica*) and explained them on the basis of a timing irregularity, as the formation of the hollow spindle is correlated with differences in spiralization. Here this is not the case; the large size of the cell and also the fact that each diplobivalent has two separately orientated centromeres and therefore will experience a greater polar repulsion may be responsible for the formation of a hollow spindle.

At late metaphase or early anaphase the chromatid structure of the diplobivalents becomes clearer and the centromeres divide. The diplobivalents of figure 1 *c*, *d*, *e*, are from such cells. This division takes place in such a way as to separate pairs of sister chromatids, so that the two chromatids attached to one centromere are both either cross-over or non-cross-over. The direction of division of the centromere is already laid down while it is still functionally a unit. Later each of these centromeres divides again to separate the sister chromatids. Thus at late anaphase the diplochromosome structure is completely lost, except that the four similar chromatids from one diplobivalent remain in the same region of the spindle. In figure 3 groups of four *M* chromatids can easily be picked out.

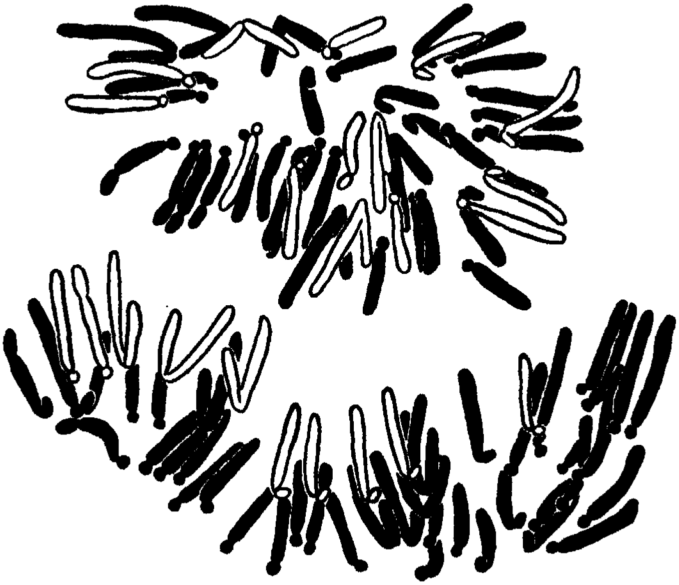

FIGURE 3. Mid-anaphase cell. The diplobivalents have broken up into their separate chromatids. There are fifty at the lower pole and forty-six at the upper. × 1200.

The anaphase itself takes place in two different ways. Usually the anaphase movement only starts after the second centromere division so that the chromatid products of the pollen-grain resting stage pass to opposite poles. This type of separation is illustrated in the upper half-bivalent in the diagram (figure 4). Sometimes, however, the anaphase movement starts immediately after the first centromere division so that the products of the pachytene chromatid division pass to opposite poles. This type is illustrated in the lower diplochromosome in figure 4. The centromeres break up later on, the sister chromatids remaining parallel and close together. Several such pairs can be seen in figure 3.

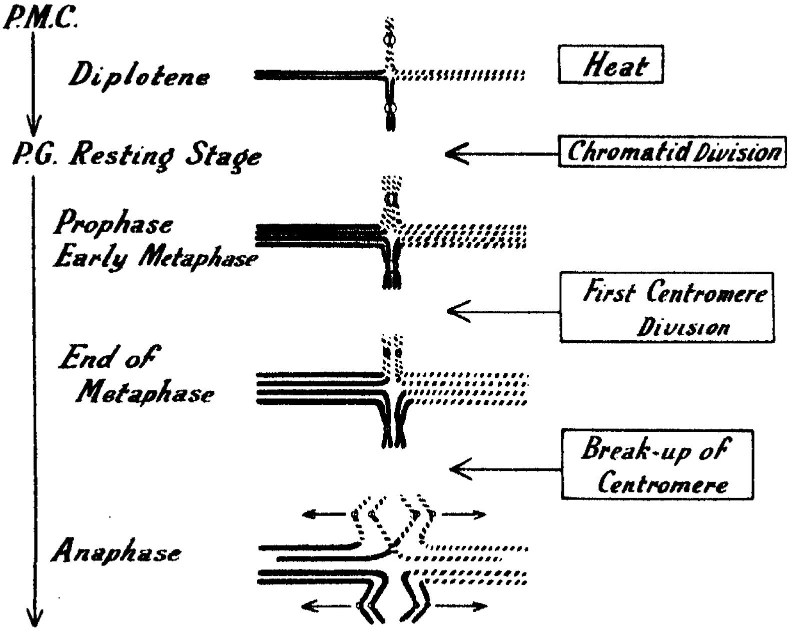

FIGURE 4. Diagram showing the origin and behaviour of diplobivalents.

This complex behaviour does not always lead to a regular separation of the chromosomes. In figure 3 there are fifty chromatids at one pole and only forty-six at the other. The separation is indeed remarkably accurate in view of the cumbrous diplobivalent structure. Probably the slight irregularity in separation is caused by a delay in one of the centromere divisions. That the divisions are not always synchronous is suggested by figure 1*e*, where one of the daughter centromeres has already divided whilst the other three in the bivalent are still single. If the anaphase movement starts before the second centromere division is complete, an unequal separation would result.

The division in the diplobivalent nuclei is thus essentially a mitotic division. It does not lead to a reduction in chromosome number. But it has one of the features of a meiotic division. The daughter nuclei will show the effects of genetic segregation following chiasma formation. The formation of diplobivalents simply delays the segregation to the pollen-grain division.

7. THE ORIGIN AND BEHAVIOUR OF DIPLOCHROMOSOMES

We have seen in the last section that the diplobivalents consist of two diplochromosomes held together by the retention of chiasmata. They are produced by the meiotic prophase nucleus relapsing into another resting stage in which a further chromatid division occurs. They are unstable and break up at anaphase to give tetraploid cells with normal chromosomes (monochromosomes in White's terminology). Their origin shows once more that the conditions necessary for centromere division are different from those under which the rest of the chromatid divides.

TABLE 2. PRESUMED OBSERVATIONS OF DIPLOCHROMOSOMES

Organism	Observer	Treatment	Tissue	Stage	Previous description
Mus	Winge 1930	Coal tar	Cancer cells	P	"Polyploid nuclei"
Locusta	White 1935	X-rays	Spermatogonia	P	Diplochromosomes
Culex	Berger 1938 *a* and *b*	Larval metamorphosis	Gut	P, M	"Multiple synapsis"
Antirrhinum	Stein 1936	Radium induced mutant	Stem meristem	M	"Pairing" in polyploid cell
Spinacia, etc.	Lorz 1937, Gentcheff and Gustafsson 1939 *a* and *b*	Old cells	Root tip	P, M	"Pairing" after two divisions in resting stage
Cucumis	Erwin 1939	Old cells	Root tip	P, M	"Polyploid nuclei"
Allium	Levan 1939	Auxines on old cells	Root tip	P, M, A	Diplochromosomes
Fritillaria	Author	Heat	Pollen grain	P, M, A	Diplochromosomes

The occurrence of diplochromosomes in mitotic divisions has been reported by several authors (see table 2). They were first observed by White (1935) in grasshoppers. He did not observe their behaviour at anaphase. Recently Levan (1939) has described their behaviour at mitosis in *Allium*. It is essentially the same as that described in the present paper. They break up at anaphase, to give at the next division tetraploid cells with normal chromosomes.

Table 2 also includes observations which were originally interpreted in a different way. Thus Lorz and Gentcheff and Gustafsson found in *Spinacia* root tips, cells with the tetraploid number of chromosomes with the homologues closely associated in two pairs (see Lorz's figures 7, 8, 9). The configurations are best interpreted as diplochromosomes. Lorz's own words are interesting in this connexion. He says of his figure 7, that "some of the chromosomes are so closely associated in pairs that they appear as chromatids". Erwin has recently (1939) found similar configurations in *Cucumis*. Similar cells also occur in tumours (Winge) and are probably to be interpreted in the same way. In all cases tetraploid cells occur with normal unassociated chromosomes. They presumably arise by the break-up of the diplochromosomes at anaphase.

The diplochromosomes in *Culex* and *Antirrhinum* are more complex. More than four chromatids can be associated at one centromere. Chromosomes with eight, sixteen, etc., chromatids may be formed (see Berger's figures 9 D and 11 F). They are *polychromosomes*. The behaviour at anaphase of the polychromosomes has not been described completely, but they occur together with cells containing the 8-ploid or 16-ploid number of monochromosomes. We must therefore assume that they break up into their separate chromatids at anaphase. This is probably the origin of the cells figured by Berger (1938*a*, figures 17, 18, 19, plate 1).

The conditions responsible for the formation of diplochromosomes are very diverse. They may be produced by the direct action of X-rays or by high temperatures. They are apparently a normal part of the developmental cycle in certain tissues as in *Culex* at metamorphosis, or in the older parts of roots. Their occurrence may also be genetically determined by a single recessive gene (*Antirrhinum*). As is so often the case in biological systems, very different external or internal agents may bring about the same effect. This is not to say that, in biological terms, the immediate causation is so varied.

The theories propounded to explain the origin of the diplochromosomes are of two main types:

(1) Hypotheses which postulate an active pairing of previously separated chromosomes.

Stein (1936), for instance, believes the diplochromosomes (as I should call them) in *Antirrhinum* to originate by pairing at the centromere. She regards it as an "autoregulative" process by which a previous doubling of the chromosomes is neutralized. This hypothesis is, teleologically, satisfying but it is difficult to understand mechanically. It cannot explain without further assumptions why the haploid number of diplochromosomes is never formed. Further, since the centromeres of the diplochromosomes break up at anaphase, no reduction can occur.

(2) Hypotheses which postulate a repeated chromatid division without a nuclear division (e.g. Levan 1939).

This is essentially the origin of the diplochromosomes in *Fritillaria Meleagris*. Here it has been experimentally proved, because the time of treatment, i.e. at prophase and also the succession of stages in the pollen mother cells and pollen grains are accurately known. Further, the characteristic chiasma structure is retained from the time of treatment to the time of observation. In the other instances we do not have such definite information but it seems probable that the various agents—heat, X-rays, physiological conditions in old cells, etc.—which induce the formation of diplochromosomes, bring about the return of a cell in early prophase to the resting stage. The centromere apparently cannot divide unless the nuclear membrane breaks down (Darlington 1937), so that at the next prophase a nucleus with the tetraploid number of chromatids but only the diploid number of centromeres is produced.

8. DIPLOCHROMOSOMES AND THE THEORY OF CHROMOSOME PAIRING

A diplochromosome consists of four chromatids passing through one centromere. As we have seen above (§ 5) they all apparently attract one another, so that the commonest arrangement of the four is in the shape of a square when viewed from the end. Very little, if anything, remains of the paired arrangement which might be expected as a result of two succesive chromatid divisions. "Sisters" do not attract one another more than "cousins". In *Culex* the position is similar. There may be bundles of up to eight or sixteen parallel chromatids showing no arrangement in pairs. These chromatids are formed by repeated division of the chromosomes without mitosis, i.e. during a prolonged resting stage (Berger 1938*b*). Gentcheff and Gustafsson (1939*b*) have also proved by means of X-ray experiments that the diplochromosomes of *Spinacia* arise in the same way. Such associations are thus associations of the products of division and may be described as *progeny pairing*.

From his observations Berger concludes that "the prophase pairing of these multiple groups furnishes additional evidence of the incorrectness of Darlington's hypothesis that the attraction between chromosomes is satisfied by union of two". It is difficult to see how such a sweeping statement is justified. Darlington's theory (1929) was based on observations on the pachytene stage of meiosis and was applied to forces acting at this stage. Pairing at pachytene whether in diploids or polyploids is limited to pairs at any one point along the chromosome. What is important is to find out when and why these rules may break down in other nuclear systems such as are provided by diplochromosomes.

Pachytene pairing differs from progeny pairing in three important respects:

(1) At pachytene, association is limited to pairs; progeny pairing is limited only by the extent of the progeny.

(2) Pachytene pairing is an active phenomenon; it brings together chromosomes previously unpaired. Progeny pairing simply maintains the status quo. Unpaired diplochromosomes remain unpaired.

(3) The pachytene chromosomes are unspiralized or nearly so (Darlington and Upcott 1939); the chromatids in diplochromosomes are separately coiled into minor spirals when pairing is at a maximum at metaphase.

A strict association in pairs as at pachytene means that pairing occurs only along one face of the chromosome; the genes are laterally as well as longitudinally specific. At mitosis the chromatids are independently coiled. It is thus impossible for the chromatids to remain paired along one face. Spiralization also has another important effect. It will convert the dorsiventral symmetry of the chromosome thread into a radial symmetry. Any remaining attraction will presumably be equal in magnitude all round the chromosome. That this must be so is proved by the gradual disappearance of relational chromatid coiling at mitosis during prophase (Upcott 1937). There is no definite attraction or pairing face but a generalized attraction round the coiled chromatid. It is, therefore, to be expected that such an attraction will not be satisfied by union in twos but, as in the diplochromosomes, may accommodate more.

Independent coiling of the chromatids in diplochromosomes is thus partly responsible for the maintenance of associations of more than two chromatids. A meiotic bivalent also consists of four chromatids, which, however, maintain a strict association in pairs after pachytene. Here the coiling relationships are more complex. The pairs of sister chromatids probably spiralize together. There may be great variation in apparent coiling relationships even in the same preparation (Darlington 1935). The techniques for the demonstration of spiral structure are effective only when they separate the coils, which can then very easily slip out of one another so that the chromatids appear separately coiled. Independent coiling of the sister chromatids at meiosis is thus probably an artefact. It appears, therefore, that the assumption of a joint major coil by the pairs of sister chromatids is a condition for the retention of the arrangement in pairs of chromatids during the first meiotic division.

The diplobivalents show that the association of chromosomes of more than two may arise not from the action of attraction but merely from the absence of repulsion, which absence depends on the type of spiralization.

9. THE STRUCTURE OF THE CENTROMERE

The diplochromosome centromere is unstable. Its instability leads to the loss of the diplochromosome structure immediately it is subjected to the action of the spindle forces, a fact which is of some significance in connexion with theories of chromatid structure. The diplochromosome is, in fact, a model of the structure which has been postulated by certain investigators (e.g. Nebel 1934) for the metaphase monochromosome. Its fate shows experimentally what happens to such a quadripartite metaphase chromosome.

As we have seen above (§ 6) the instability of the diplochromosome centromere is controlled in that the directions of the successive divisions are laid down while it still behaves as a unit on the spindle. Its lines of division follow those laid down by the successive chromatid divisions. We can explain this only if we assume that the chromatids are continued through it in some form. Its behaviour as a unit during orientation must therefore be dictated by some other structural feature. The centromere has a dual structure. Darlington (1939) from observations of misdivision of the centromere at meiosis has concluded that the centromere consists of a fluid surrounding fibres which may be said to continue the chromatid structure through the centromere. The fibres normally control its direction of division. Thus in a diplochromosome, the centromere orientates as a unit because of the continuous fluid surrounding the fibres. The directed division of the centromere proves that the fibrous elements are already divided once by the beginning of metaphase. The second fibre division takes place later, presumably just before the second fluid division as in a normal mitosis. Double fibres are not stable in the anaphase centromere.

The behaviour of the centromere in a diplochromosome is thus in direct contrast to that in a meiotic bivalent where the centromere does not break up during anaphase. We must assume that there is only a single fibre in each centromere of a bivalent. In other words, the onset of the meiotic anaphase is precocious with respect to centromere division just as the onset of the meiotic prophase is precocious with respect to chromatid division. Further, the precocity of meiosis is responsible for the capacity for co-orientation of the two centromeres in a bivalent since, as we have seen above, the centromeres with double fibres in the diplobivalents cannot co-orientate.

SUMMARY

1. Heating after pachytene has no effect on the frequency or proximal localization of chiasmata in the pollen mother cells of *Fritillaria Meleagris*.

2. Continued high temperatures during the meiotic divisions have two effects:

(*a*) The separation of the chromosomes is followed by the development of a restitution nucleus giving diploid or tetraploid pollen grains with normal chromosomes.

(*b*) The meiotic nuclear divisions are completely suppressed, so that a diplotene nucleus lapses directly into a pollen-grain resting nucleus. The pollen-grain chromatid division takes place to give, at the metaphase of the pollen-grain mitosis, diplochromosome bivalents consisting of eight chromatids.

3. The diplobivalents retain the chiasma structure of the meiotic bivalents except that two chromatids replace one.

4. Each chromatid in a diplobivalent has a simple mitotic coil and is coiled independently of the others. There is no well-defined attraction in pairs, each arm consisting of two parallel chromatids at full metaphase. Thus joint-coiling of the major spirals is a condition of the retention of the arrangement of chromatids in pairs at first metaphase of meiosis.

5. Each half-diplobivalent has a single centromere with the four chromatids passing through it. The centromeres orientate separately. Thus the co-orientation of centromeres at meiosis depends on the internal structure of the centromere, not on chiasma formation.

6. At anaphase two successive divisions take place in the centromere. The first separates the products of the pachytene division; the second the products of the pollen-grain resting stage division. The mitosis will thus give two tetraploid daughter nuclei with normal chromosomes. They will differ by the segregation of the products of crossing-over at the suppressed meiosis.

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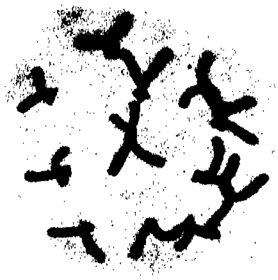


Figure 5

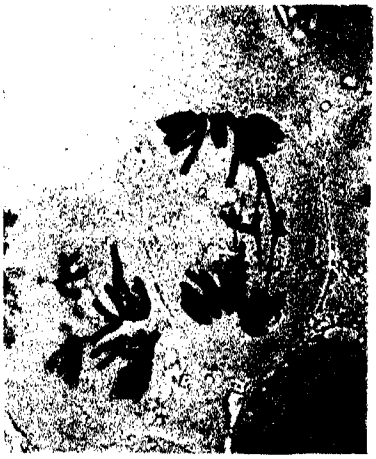


Figure 6

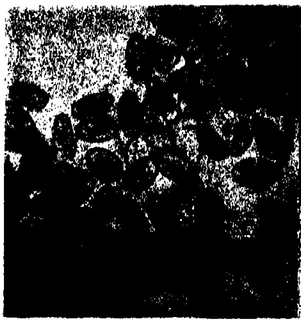


Figure 7

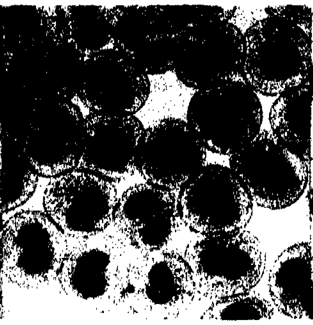
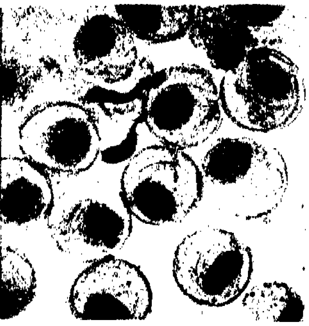


Figure 8



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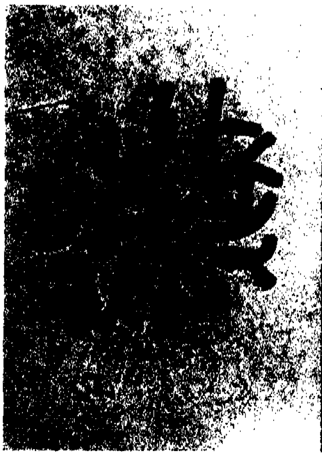

Figure 10

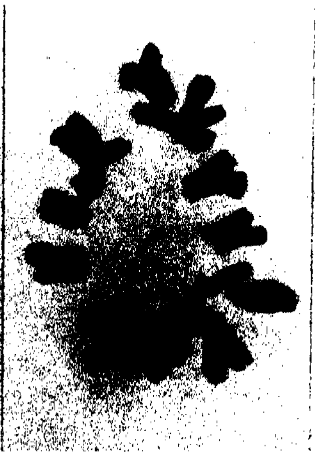

Figure 11

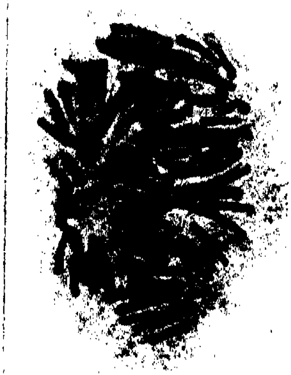

Figure 12

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Description of Plate 13

Figure 5. First metaphase of meiosis in normal *Fritillaria Meleagris*. The cell has been flattened and the chromosomes spread out in smearing. × 500.

Figure 6. First anaphase from plant heated 2 days at 30° C. The chromatids are sticking together. × 500.

Figure 7. Normal tetrad pollen from control plant. × 125.

Figure 8. Binucleate monad pollen grains from plant heated 2 days at 30° C. The second meiotic division was completely suppressed. × 125.

Figure 9. Pollen grains from pollen mother cells in which both meiotic divisions were suppressed. × 125.

Figure 10. Early metaphase in pollen grain with diplobivalents. The centromeres are undivided. The nucleolar constriction is visible in the bivalent at 11 o'clock. × 500.

Figure 11. Diplobivalents at the beginning of anaphase. × 500.

Figure 12. Mid-anaphase from diplobivalents. × 500.

The reactions of the urinary bladder of the cat under conditions of constant volume

By the late J. MELLANBY, F.R.S. AND C. L. G. PRATT

INTRODUCTION

A description of the reactions of the bladder of the cat under conditions of constant pressure has already been given (Mellanby and Pratt 1939). Numerous workers have obtained records of variations in pressure in the bladder of the cat under approximately isometric conditions, whilst examining the influence of the central nervous system on the process of micturition. However, a clear distinction between the isotonic and isometric states with respect to the bladder has not been generally observed. The experiments described in this paper were undertaken in order to study the effects of the changed conditions set up in the bladder when the volume of the bladder is kept fixed, especially after a period during which the pressure is kept fixed, and the volume allowed to fluctuate. In particular, the striking response to a sudden transition from the isotonic to the isometric state has been investigated.

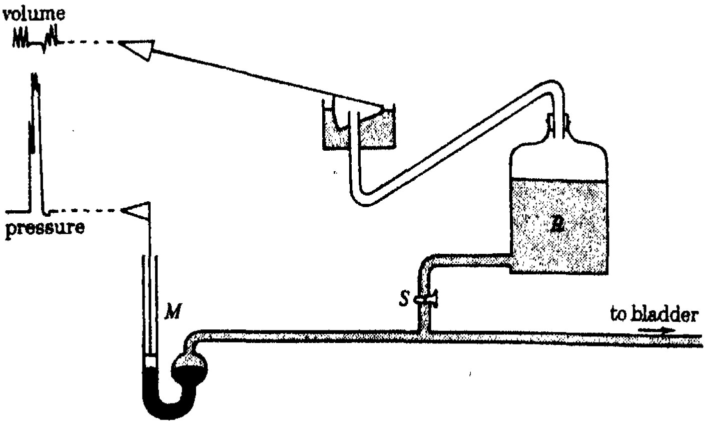

FIGURE 1. Diagram of essentials of apparatus. *M*, mercury manometer; *R*, adjustable reservoir; *S*, stopcock.

TECHNIQUE

The apparatus used was that described in the previous paper, with the addition of a narrow-bore recording manometer filled with mercury. The general arrangement is illustrated in figure 1. With the stopcock open and the contents of the bladder therefore in communication with the contents of the reservoir, the manometer records the fixed hydrostatic pressure imposed upon the bladder by the level of the fluid in the reservoir. Closing the stopcock disconnects the reservoir from the bladder, and the manometer then records any fluctuations in pressure in the bladder. With such an arrangement it is possible to make a practically instantaneous change from the isotonic to the isometric state, or vice versa, without disturbing the preparation.

Cats under chloralose or urethane anaesthesia were used for most of the experiments. A series of experiments was also performed upon decerebrate cats.

RESULTS

I. *The isometric contraction*

(*a*) *General features.*

The following observations were made on preparations which had shown a well-developed regular isotonic rhythm for at least ½ hr. before being made isometric. This was taken as evidence that the effect of the volatile anaesthetic, used for purposes of induction, had passed off.

If the bladder is made isometric at the moment when an upstroke of the isotonic rhythm is about to be inscribed, i.e. when the volume of the bladder is about to diminish slightly, the immediate response is a contraction producing a rise of hydrostatic pressure of striking proportions (figure 2). The response follows without any detectable latent period. The pressure rises rapidly to somewhere between 50 and 100 mm. Hg and may be maintained at that level with relatively small irregular fluctuations for a period of 30 sec. to 1 min., after which it usually declines rapidly to approximately zero, irrespective of the initial hydrostatic pressure during the execution of the isotonic rhythm.

The precise pressure reached during the isometric contraction varies considerably in different preparations, but after repeated elicitations at the beginning of an experiment, tends to reach a steady high level. The highest value recorded has been 160 mm. Hg.

After the isometric contraction, the hydrostatic pressure may remain indefinitely at zero, or a series of similar spontaneous contractions may continue at intervals of about 1 min. (figure 3).

(*b*) *Relation to the isotonic rhythm.*

If the bladder is made isometric at the moment when a downstroke of the isotonic rhythm is about to be inscribed, i.e. when the volume of the

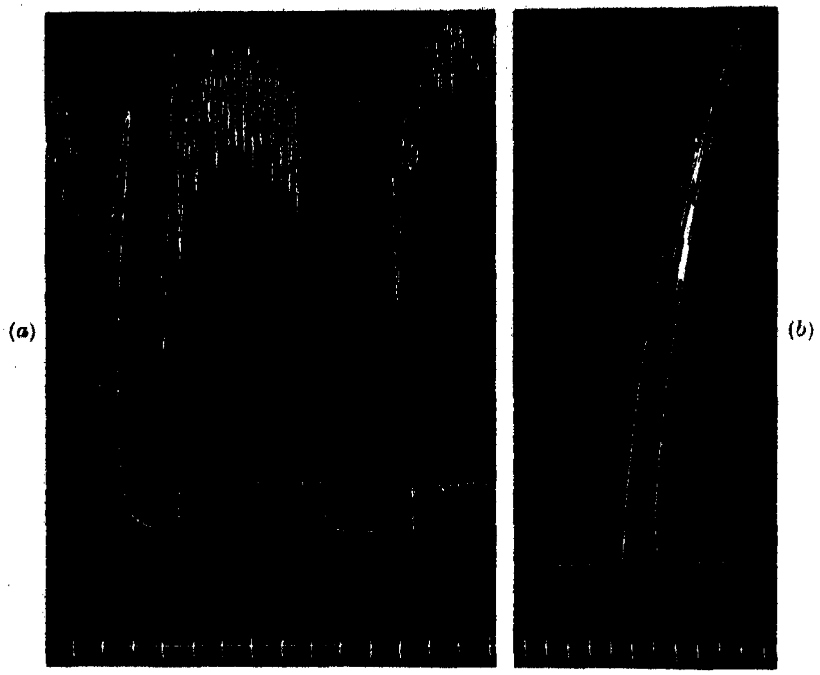

FIGURE 2. (*a*) Upper record, isotonic rhythm; lower record, isometric contraction and isometric relaxation. (*b*) Spontaneous isometric contraction in quiescent bladder. Twitch-like contractions superimposed on pressure record due to brief contractions of abdominal and other muscles. Time in 30 sec.

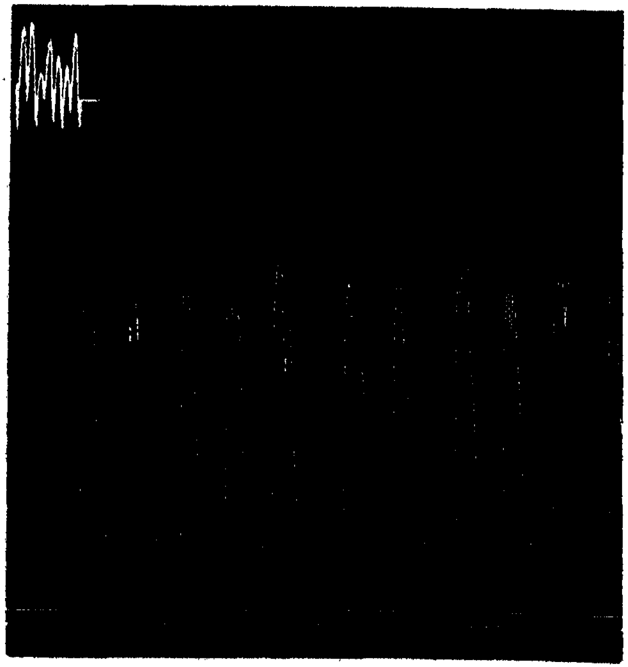

FIGURE 3. Slow isometric rhythm. Pressure falls to zero between each contraction. Time in 30 sec.

bladder is about to increase slightly, the pressure in the bladder falls at once to zero, without executing an isometric contraction (figure 2).

It is possible to elicit the isometric contraction at points in the isotonic rhythm other than that at which an upstroke is about to be inscribed. Figure 4 shows the result of a typical experiment in which the bladder was suddenly made isometric at different points of the isotonic rhythm.

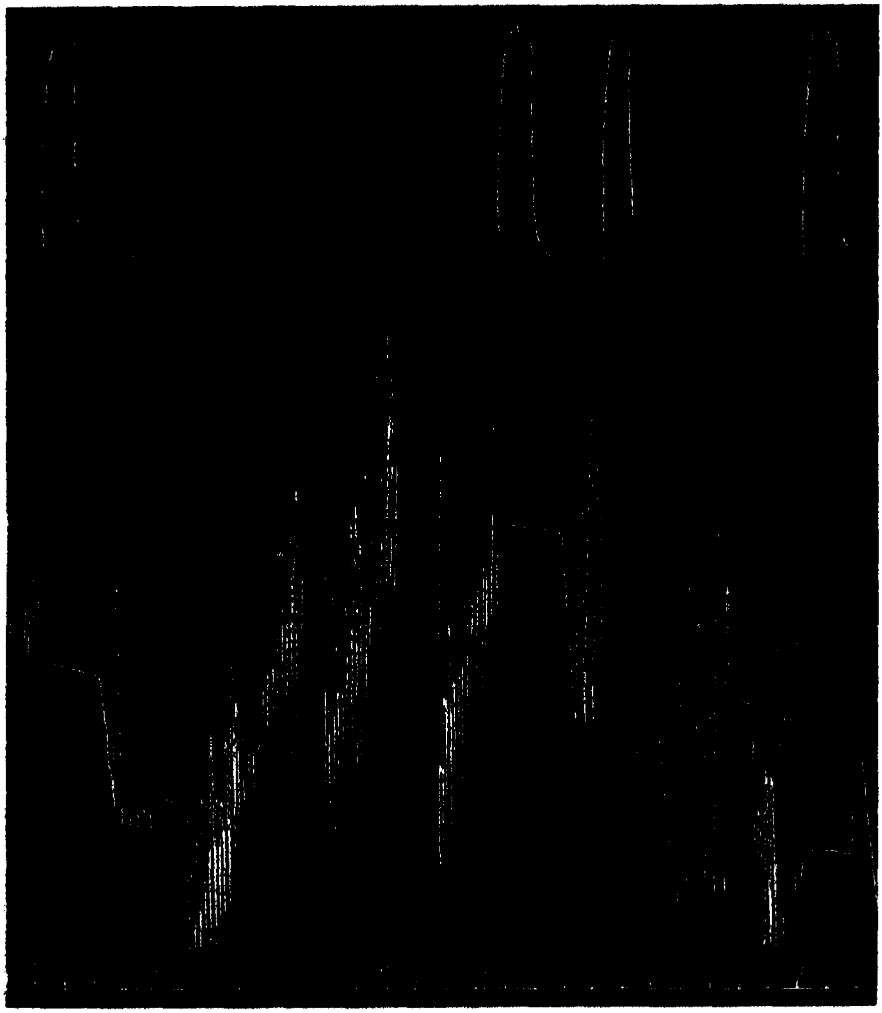

FIGURE 4. Relation between isotonic rhythm and isometric contraction. Upper record, isometric; lower, isotonic. Isometric contraction follows when stopcock closed at *A*, *E*, *F* and *H*; relaxation when stopcock closed at *B*, *C*, *D* and *G*. Time in 10 sec.

If the change from the isotonic to the isometric state is made at any moment corresponding to the last third of the downstroke or the first two-thirds of the upstroke of the isotonic rhythm, then under ordinary circumstances the isometric contraction is immediately evoked. Transfer from isotonic to isometric conditions during the inscription of the last third of the upstroke or the first two-thirds of the downstroke invariably leads to isometric relaxation to zero pressure, which may or may not be interrupted after a variable interval by an isometric contraction or the beginning of an isometric rhythm (figure 5).

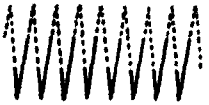

FIGURE 5. Diagram of relation of isometric contraction to isotonic rhythm. Contraction follows closure of stopcock at any point during continuous part of rhythm; relaxation if stopcock closed during broken part.

(c) *Spread of excitation to the voluntary muscles.*

In the decerebrate cat, or in the cat so lightly chloralosed that limb reflexes are brisk, the production of the isometric contraction in the usual way is accompanied by a brief series of jactitations which are recorded as twitches at the height of the contraction (figure 2). These movements are due to contractions of the muscles of the diaphragm, the abdominal wall, the shoulder girdle and the pelvic girdle. Together they effect a transitory augmentation of the hydrostatic pressure in the bladder. They are only seen when the isometric contraction has reached its maximum, and often not until it is beginning to decline. Barclay and Franklin (1937) observed a similar viscerosomatic reflex on manual compression of the bladder, but they did not show, though they assumed, that the contraction of diaphragm and abdominal wall in turn increases the intravesical pressure.

II. *The influence of anaesthetic agents; variations in rhythm*

The pressure reached during the isometric contraction depends to some extent on the depth of anaesthesia. A well-established contraction in the chloralosed cat may be temporarily abolished by the administration of ether, reappearing as the effect of the ether wears off.

The post-contraction quiescent state is more regularly seen under urethane, whereas with chloralose an isometric rhythm is more usual. When the post-contraction quiescent state appears under chloralose, it is not so persistent as with urethane, generally lasting for not more than 20 min., and then giving place to the isometric rhythm.

Occasionally, under chloralose, a different type of rhythm is seen. In this case the instantaneous change to isometric conditions evokes a series of short contractions occurring at a frequency as great as that of the isotonic rhythm, reaching a pressure of not more than 20 mm. Hg. This type of rhythm either persists indefinitely, or ceases in 1–2 min., whereupon the hydrostatic pressure falls immediately to zero, and the bladder assumes the quiescent state seen after the large slow contraction. Such a rapid rhythm of low pressure is generally observed in cats in which the calculated dose of chloralose has produced deeper level of anaesthesia than is normally the case. Sometimes the two types of rhythm are combined, large slow contractions interrupting at intervals a continuous series of smaller, more rapid contractions (figure 6).

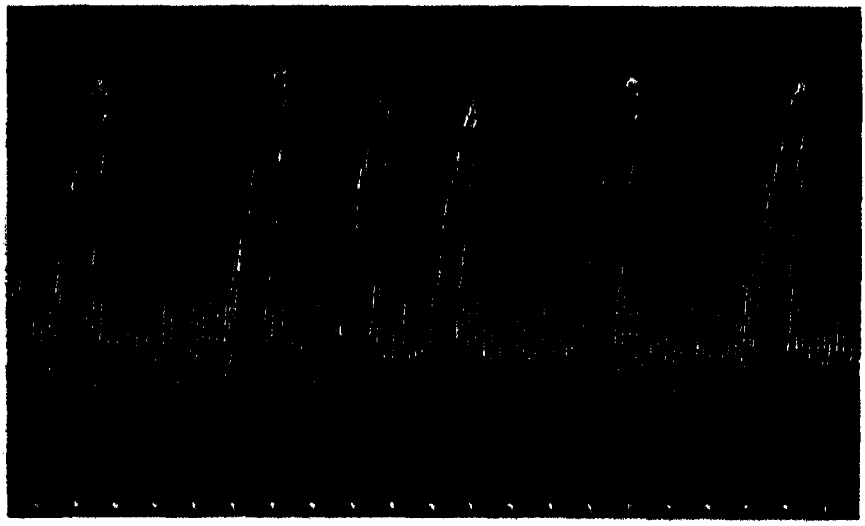

FIGURE 6. Combined slow and rapid isometric rhythm. Time in 30 sec.

III. *The influence of the nervous system*

A. *The hypogastric nerves.*

(*a*) *Section.* The isometric contraction is unaffected by section of both hypogastric nerves, nor does section of these nerves appear to modify the activity of the quiescent or spontaneously contracting bladder under isometric conditions. This is unexpected, since section of the hypogastric nerves generally augments the amplitude and diminishes the frequency of the isotonic rhythm.

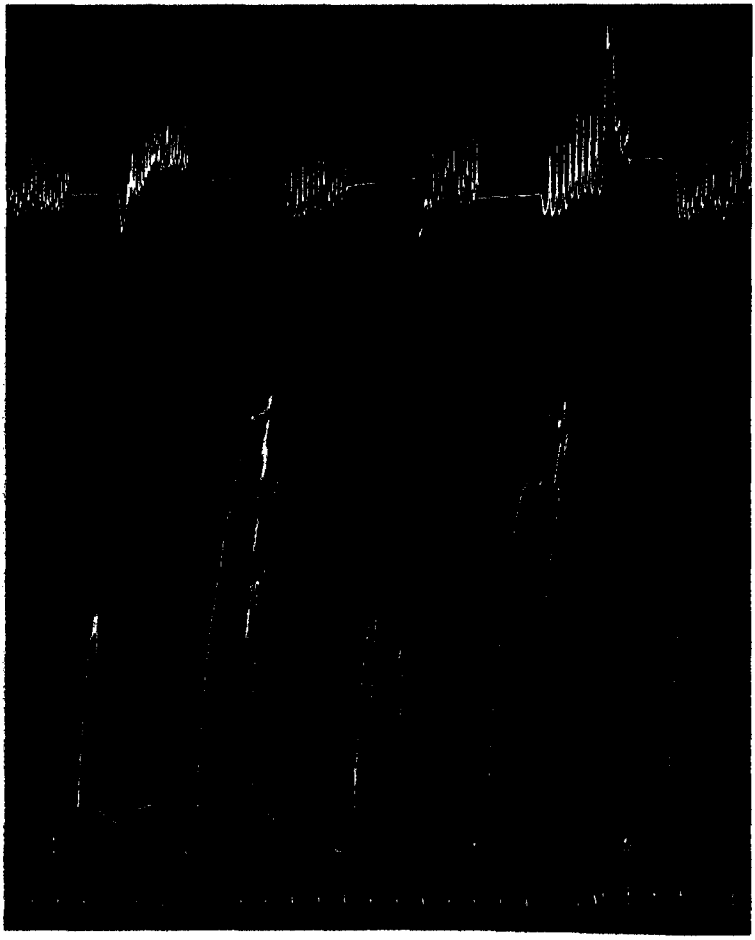

FIGURE 7. Stimulation of hypogastric nerves. Upper record, isotonic rhythm; lower, isometric contraction. At 1 and 3, stimulation commences at the moment of making isometric; at 2 and 4, normal isometric contraction; at 5, contraction is produced by making isometric during the course of hypogastric stimulation under isotonic conditions. Time in 30 sec.

(*b*) *Stimulation*. In the quiescent bladder, stimulation of the hypogastric nerves produces a rise of pressure similar to that of the spontaneous isometric contraction, but of smaller degree. There is no appreciable latent period.

It is generally agreed (Gruber 1933) that the hypogastric nerves carry motor impulses to the bladder in animals. McRea and Macdonald (1936) have shown the same to be true in man. In the present experiments it was observed that the contraction produced by hypogastric nerve stimulation was always inferior to that produced by stimulation of the nervi erigentes, in spite of the fact that the hypogastric nerves supply approximately the same number of fibres to the vesical plexus as do the nervi erigentes (Moseley 1936).

It is possible to demonstrate the inhibitory influence of activity of the hypogastric nerves on the isometric bladder in two ways. Firstly, if stimulation begins simultaneously with the elicitation of an isometric contraction, then the height and sometimes duration of that contraction are less than normal (figure 7).

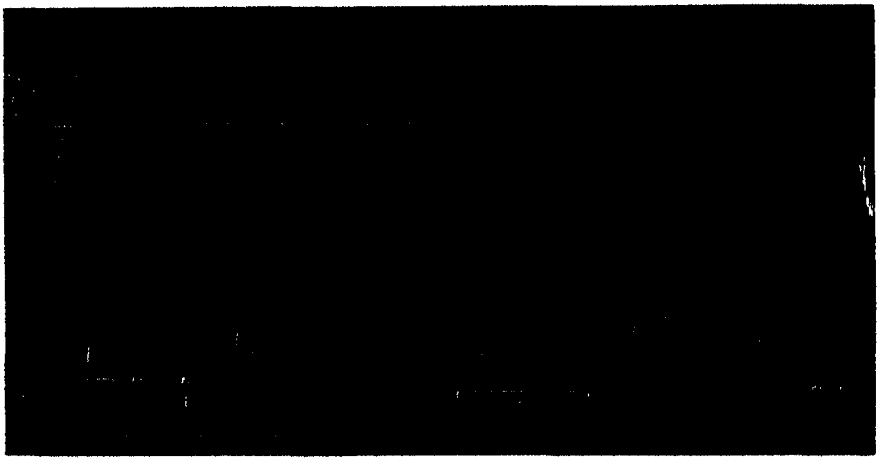

FIGURE 8. Stimulation of hypogastric nerves, 1, normal isometric contraction; 2, 3, 4, repeated hypogastric stimulation; 5, longer hypogastric stimulation inhibits the rhythm; 6, 7, repeated hypogastric stimulation. Time in 30 sec.

Similarly, if stimulation of the hypogastric nerves is begun whilst the bladder is still isotonic, and the isometric contraction is elicited during the period of stimulation, the contraction is also sub-normal. Secondly, an isometric contraction produced as a result of stimulation of the hypogastric nerves is followed by a period of diminished excitability of the bladder. This is best seen during a spontaneous isometric rhythm. Stimulation of the hypogastric nerves promptly interpolates a sub-normal contraction, which is followed by a period of quiescence for as long as the stimulation is continued (figure 8). Furthermore, a repetition of the stimulation within 2 min. of the cessation of the first, produces a much smaller contraction.

B. *The nervi erigentes.*

(*a*) *Section.* Section of the nervi erigentes abolishes the isometric contraction or rhythm, just as it deranges the isotonic rhythm. It has been impossible to demonstrate any return of activity during periods up to 6 hr. after section of the nerves, even though there has been a moderate degree of recovery of the isotonic rhythm. It is therefore possible that the isometric contraction produced by instantaneously changing the state of the bladder from isotonic to isometric is reflex in nature. Experiments are being performed to decide whether this is so, and the results will be reported in another communication.

(*b*) *Stimulation.* Stimulation of the nervi erigentes either singly or together produces a great rise in pressure which is sustained for as long as the stimulation continues. The post-contraction excitability is not depressed as after stimulation of the hypogastric nerves. Experiments were undertaken to determine whether it is possible to diminish the pressure reached during stimulation of the nervi erigentes by simultaneous stimulation of the hypogastrics, but this appears not to be so.

IV. *The action of adrenaline*

The response of the isometric bladder to adrenaline depends upon the state of activity of the bladder at the time of injection.

(*a*) *Action on the quiescent bladder.*

In the quiescent bladder, the intravenous injection of adrenaline is followed after an interval of 20–50 sec., if the dose is not too great, by a rise of pressure similar in height and duration to the isometric contraction. A contraction of this nature is readily obtained with doses between 0·01 and 0·1 mg. With doses of 1 mg. contraction is sometimes seen, but the commonest response is a period of quiescence which may be prolonged for several minutes.

(*b*) *Action on the rhythmic bladder.*

Moderate doses of adrenaline influence the isometric rhythm in a characteristic way. A contraction in process of inscription may be foreshortened in height and time, or the normal interval between one contraction and the next may be prolonged. In either case the next following contraction is of greater amplitude than the normal. This effect is especially well seen during the performance of an isometric rhythm of high frequency but low amplitude. In this case the contraction which follows the injection of adrenaline is prolonged and of high pressure (figure 9).

Whatever the state of activity of the bladder, an isometric contraction induced by adrenaline is always preceded by a very long latent period. The actual length of this interval appears to depend to some extent on the dose of adrenaline employed.

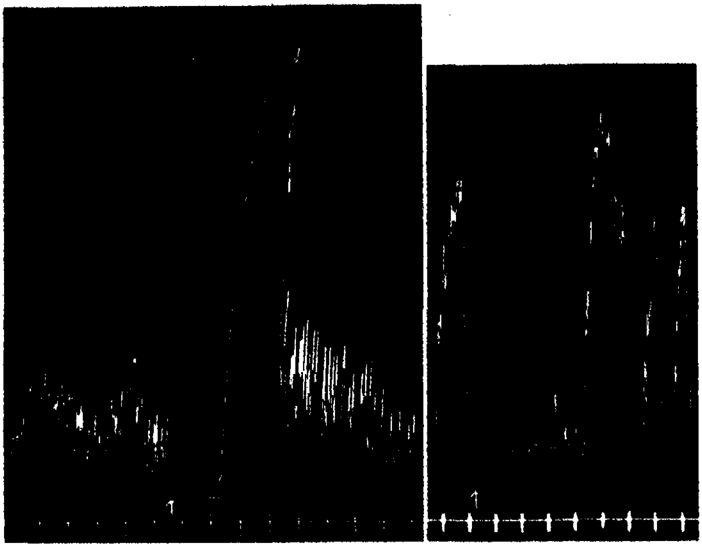
(*a*) (*b*)

FIGURE 9. (*a*) Adrenaline 0·1 mg. on rapid rhythm; strong contraction after latent period. (*b*) Adrenaline 0·1 mg. on slow rhythm; augmentation of next contraction after latent period. Time in 30 sec.

V. *The action of acetylcholine*

In the quiescent bladder, acetylcholine in doses of 0·01–1 mg. produces a contraction with no appreciable latent period (figure 10).

The injection of acetylcholine during the execution of the isometric rhythm augments the height of the contraction next following the injection. The interval between this augmented contraction and the subsequent contraction may be prolonged by 10–60 sec. This effect is best seen when acetylcholine is given while the rhythm is small and rapid. In that case the augmentation is very great in proportion to the amplitude of the fundamental rhythm, and the subsequent period of quiescence is maximal. Eserine potentiates the action of acetylcholine.

VI. *The action of atropine and ergotoxine*

Atropine rapidly abolishes the isometric rhythm (figure 11). This is in accord with the effect of atropine on the isotonic bladder where it diminishes the amplitude of the rhythm and causes dilatation.

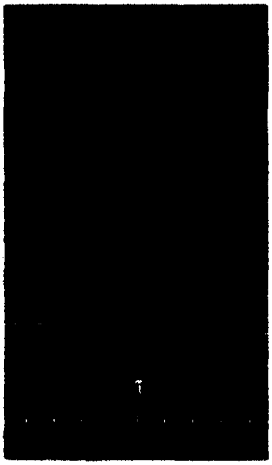

Figure 10. Acetylcholine 0·1 mg. on quiescent bladder. Prompt contraction. Time in 30 sec.

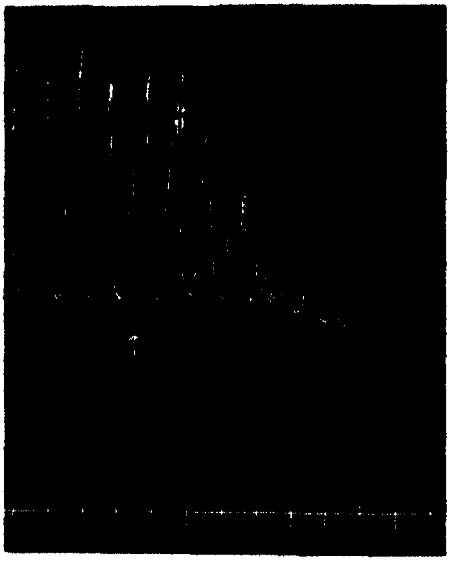

Figure 11. Atropine on isometric rhythm. 0·2 mg. intravenously. Time in 30 sec.

Atropine prevents the contraction produced by acetylcholine or by adrenaline. Administered subcutaneously in amounts insufficient to abolish the isotonic rhythm, it prevents the production of the characteristic contraction normally resulting when the bladder is made isometric (figure 12).

Ergotoxine in small doses does not inhibit the contraction, or the augmentation of contraction, produced by adrenaline, but in large doses it produces the condition of fixity described in the previous paper. In this condition it is impossible to elicit the isometric contraction or to demonstrate the effects of adrenaline or acetylcholine.

Discussion

The activity of the isometric bladder differs in several respects from that of the isotonic bladder in the chloralosed cat. Without the stimulus of an artificially imposed constant hydrostatic pressure, the bladder tends to adopt a condition of quiescence at zero pressure, relieved at relatively infrequent intervals by bursts of activity resulting in the characteristic temporary rise of pressure which has been described. In the isotonic bladder, on the other hand, contraction and relaxation alternate without intervening periods of inactivity. In the isotonic preparation the constant fluctuations of volume are relatively small, but the intermittent variations of pressure of the isometric bladder are very large, rising to far beyond the hydrostatic pressure which would result in micturition were the urethra not tied. Moreover, the execution of an isotonic contraction occupies

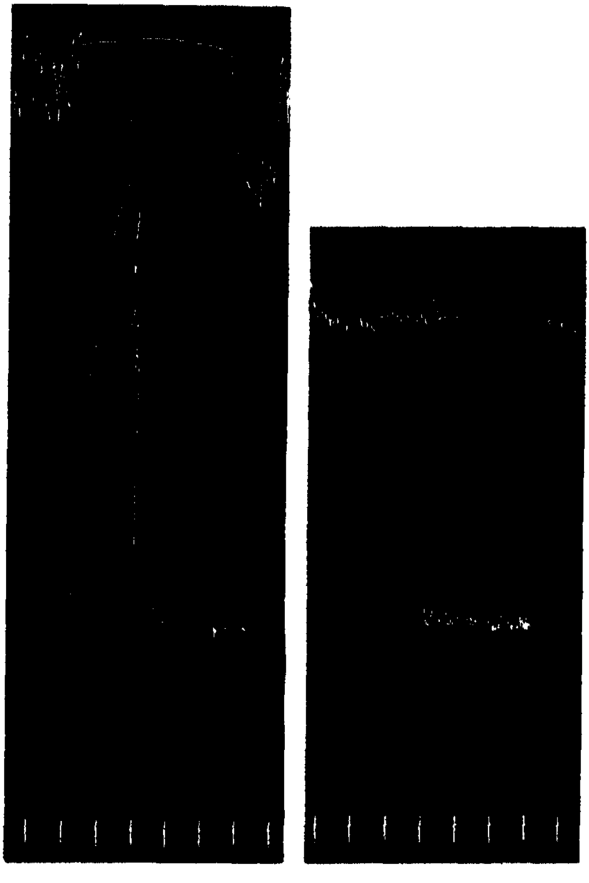
(*a*) (*b*)

FIGURE 12. Atropine on isometric contraction. (*a*) Before, (*b*) 40 min. after 2 mgm. atropine sulphate subcutaneously. Time in 30 sec.

about 10 sec., whereas an isometric contraction is typically sustained for more than 60 sec. Lastly, a regular isotonic rhythm is established some time before any activity under isometric conditions can be detected. The mechanism involved in the isometric contraction is evidently more susceptible to the influence of the ether used for induction of anaesthesia than is that responsible for the isotonic rhythm. This is borne out by the fact that the isometric activity is abolished more readily than is the isotonic rhythm by readministering ether when both have become well established. The isometric activity is even less resistant than the isotonic rhythm to the action of atropine.

The isometric contraction, with its characters of great rise of pressure, maintained contraction, and subsequent relaxation to zero pressure, evoked by making the isotonic bladder isometric at a suitable moment, has not previously been described. Since it is abolished by dividing the nervi erigentes, though not affected by section of the hypogastrics, it is probably reflex in origin and appears to be related to the micturition reflex. It has long been known that the elicitation of the micturition reflex is related to the hydrostatic pressure of the bladder and not to its volume. Mosso and Pellacani (1882) observed that in a trained unanaesthetized dog, the attempt to micturate always occurred at about 20 cm. H_2O, whatever the volume of the bladder, when warm water at gradually increasing pressures was run into the bladder. Guyon (1900), also in the dog under curare, showed that a bladder volume could be found at which the addition of a small additional volume, not of itself raising the intravesical pressure more than 10 cm. H_2O, provoked a sudden rise of hydrostatic pressure to about 90 cm. H_2O.. Barrington (1914) described a similar phenomenon, which he considered to be artificially produced micturition, in the decerebrate cat. On the other hand Elliott (1907), in the cat under A.C.E., was able to produce the Mosso effect by simple increase of volume. In one experiment, increasing the volume from 10 ml. to 50 ml. raised the pressure only from 0·5 cm. to 4 cm. At the latter pressure there was an oscillatory rise to 60 cm., which was the top of the manometer, and at that pressure the bladder discharged 40 ml. None of these investigators observed a rise of pressure comparable with that seen during the isometric contraction, nor were any of them able to evoke the micturition reflex under strictly isometric conditions, that is, without introducing additional fluid into the bladder.

The isometric contraction may depend upon the presence of stretch receptors in the wall of the bladder. Stella (1934) demonstrated the existence of such receptors in the frog and Evans (1936) extended the observation to the cat. According to Evans, a sudden reduction of hydrostatic pressure in the bladder momentarily abolishes the otherwise continuous electrical activity in the nervi erigentes. There is therefore a close relation between variations in intravesical pressure and the presence of afferent impulses in the nervi erigentes. The anatomical basis for this was established by Langley and Anderson (1894) who found that about one-third of the fibres of the nervi erigentes are afferent. Talaat (1937) examined the action potentials in the nervi erigentes and the hypogastric nerves under chloralose and other anaesthetics. He found that the bladder wall contains two groups of afferent nerve endings. The first group are stimulated by contraction of the muscle of the bladder, and these nerve endings adapt rapidly; the second group respond to changes of intravesical pressure and adapt more slowly. The nerve endings of the second group vary considerably in their threshold of excitation, some beginning to respond at a pressure of 8 cm. H_2O whilst others do not respond until a pressure of 25 cm. H_2O is reached. The production of the isometric contraction cannot be related with certainty to the activity of these various types of afferent nerve ending, especially since an analysis of their function is available only for the dog. Evans (1936), quoted above, did not examine in detail the afferent impulses from the bladder of the cat. Nevertheless, the production of an isometric contraction by making the bladder isometric at the moment when an isotonic contraction has begun, or is imminent, indicates that the bladder wall of the cat possesses, like that of the dog, afferent nerve endings, which are stimulated by contraction of the muscle of the bladder. Further, it is possible that the gradual attainment of maximum pressure by a series of increments separated by slight pauses may be correlated with nerve endings of the second type which are excited serially in groups as the rising intravesical pressure reaches their several thresholds of excitation. However, there remains the difficulty of explaining, in such terms of reflex action, the spontaneous appearance of an isometric contraction or rhythm in the quiescent bladder at zero pressure. Under such conditions there is no change of state artificially imposed upon the bladder muscle to account for the beginning of the process, which must therefore be attributed to the spontaneous activity of either the muscle or the nervous system. It is significant that a spontaneous isometric contraction or rhythm is less often obtained under urethane than under chloralose. In the doses used, the former drug depresses the reflex activity of the whole preparation considerably more than does chloralose.

The viscerosomatic reflex obtained in the decerebrate animal or under light chloralose anaesthesia has an obvious usefulness to the organism.

The contraction of diaphragm, abdominal muscles and limb muscles momentarily raises still farther the already high intravesical pressure and so provides the maximum possible force in the attempt to overcome the obstruction to the urethral passage. The reflex is not called into play until the intrinsic muscle of the bladder has proved unable to force the sphincter.

The action of adrenaline on the isometric bladder calls for comment. It is much easier to demonstrate contraction than relaxation as the result of the intravenous injection of this drug, but however small the dose, provided it is effective, contraction is preceded by a long latent period. This is in strong contrast to the action of acetylcholine, which produces a prompt contraction, and to the action of adrenaline on the isotonic bladder, where the effect can be reversed by appropriate variation of dosage. It appears that the isometric contraction produced by adrenaline is not the immediate result of the action of the drug directly on the muscle fibres or on adrenergic nerve endings, but that some process is set in motion which leads, at the end of the latent period, to a co-ordinated contraction indistinguishable from the post-isotonic isometric contraction. What events occur in the neuromuscular mechanism of the bladder during this latent period is unknown.

SUMMARY

Instantaneous change from constant pressure to constant volume conditions produces either an isometric contraction or a state of quiescence at zero pressure, according to the phase of the isotonic rhythm at which the change is made. The isometric contraction may be followed by a state of quiescence at zero pressure for an indefinite period, or by a rhythmic series of similar contractions. The bladder nearly always relaxes to zero pressure in the intervals between isometric contractions. The isometric contraction differs from an isotonic rhythmic contraction in several particulars, which are described. The hydrostatic pressure reached during an isometric contraction may be more than 100 mm. Hg. The isometric contraction is probably reflex in nature.

Under light chloralose anaesthesia, and in the decerebrate cat, the height of the isometric contraction may be accompanied by twitches of the diaphragm, abdominal muscles and limb muscles. Division of the hypogastric nerves does not modify the isometric contraction. Stimulation of the peripheral end of the cut hypogastric nerves causes a submaximal isometric contraction, followed by a period of diminished excitability. Division of the nervi erigentes abolishes the isometric contraction. Stimulation of the

peripheral ends of the cut nervi erigentes causes a maximal isometric contraction.

Adrenaline produces contraction, after a long latent period, similar to the isometric contraction. Acetylcholine produces a prompt contraction similar to the isometric contraction. Atropine abolishes the isometric contraction more readily than it destroys the isotonic rhythm.

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Calcium and blood coagulation

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INTRODUCTION

Green (1887) was the first to show that "calcium sulphate is instrumental in bringing about coagulation when added to a plasma which shows little or no tendency to clot, and that coagulation in its absence is almost or quite prevented". In 1890 Arthus and Pagès extended these observations and prepared non-coagulable mammalian blood by the addition to it of 0·1 % potassium oxalate, or of 0·15 % sodium fluoride.

The part played by calcium in the coagulation of the blood has been the subject of many hypotheses. Pekelharing (1896) assumed that thrombase is a calcium compound of nucleoprotein (prothrombase*) and that the action of thrombase* consists in transferring calcium to fibrinogen and thereby converting it to fibrin. Morawitz (1904) and Fuld (1904) stated that both calcium salts and thrombokinase were equally concerned in the conversion of prothrombase to thrombase. On Howell's hypothesis (Howell 1925) calcium alone converts prothrombase to thrombase. In recent times the original view of Pekelharing has been put forward in modified form by Mills (1926) who considers that thrombase is a complex of calcium, cephalin and prothrombase.

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* The terms thrombase and prothrombase are here used for substances also known as thrombin and prothrombin.

The properties of calcium in plasma and the action of calcium in (*a*) the generation of thrombase from prothrombase, and (*b*) coagulation of plasma by thrombase, have been investigated, and the results are presented in this paper.

Experimental methods

The plasma used in many of the experiments was prepared from cockerel's blood by the method described by Delezenne (1897). As a test solution for thrombase, oxalated ox plasma (containing 0·2% potassium oxalate) was used. The prothrombase and thrombase preparations were made by the methods previously described by Mellanby (1930, 1933). In many of the experiments, the dried venom of Russell's viper (*Daboia russellii*) was used as the source of the thrombokinase. This venom contains a remarkably potent thrombokinase of which 0·005 mg. coagulates 1 ml. of fowl plasma in 1 min., whilst it has no action on oxalated fowl plasma. The potency of the venom as a source of thrombokinase makes it preferable to tissue extracts in quantitative work.

Calcium was estimated by the method of Kramer and Tisdall (1921).

Results

1. *Calcium in serum and plasma*

It is generally held that serum and plasma have approximately the same concentration of calcium. On the other hand, Stewart and Percival (1928*a*) found that the calcium value of plasma was 10–20% greater than that of serum. Possibly when the serum is expressed from coagulated blood, the corpuscles may adsorb calcium during the time required for the separation. Alternatively the preparation of plasma from blood by the use of anticoagulants such as heparin may upset the calcium equilibrium when coagulation is effected by the required agent. The use of stable plasma from fowl blood is free from both of these objections. Four ml. of fowl plasma were coagulated by the addition of 0·01 mg. of *Daboia* venom.

The calcium content of the original plasma and the serum expressed from the coagulated plasma were: plasma, 10·7 mg. %; serum, 10·6 mg. %. These figures are identical within the limits of experimental error.

2. *Calcium in the formation of fibrin from fibrinogen*

The identity of the calcium figures for plasma and serum indicate that calcium does not combine with fibrinogen in the formation of fibrin, a view which is in accord with the observation that a calcium-free thrombase solution actively coagulates plasma containing 0·2 % calcium oxalate. It is possible, however, that apart from entering into specific combination with the interacting substances, calcium accelerates the action of thrombase on fibrinogen. This possibility was investigated on fowl plasma.

The relation of calcium to the coagulation of plasma by thrombase.

The first series of experiments was made on fowl plasma to which varying quantities of oxalate had been added. After 30 min. a constant quantity of thrombase was added to each tube and the times of coagulation noted (see table 1).

TABLE 1

Plasma	H_2O	N/40K_2Ox	Thrombase (0·1 %)	Coagulation time
0·5	0·4	0	0·1	30
0·5	0·3	0·1	0·1	30
0·5	0·2	0·2	0·1	40
0·5	0·1	0·3	0·1	45
0·5	0	0·4	0·1	45

Quantities in ml.; time in sec.

In a second series of experiments fowl plasma was dialysed against distilled water and the calcium reduced thereby from 10·8 mg. % to 3 mg. %. The precipitated fibrinogen was put into solution by the addition of sodium chloride (0·5 %). Varying quantities of calcium chloride were then added, and after 30 min. a constant quantity of thrombase was added to each tube as before. The following results were obtained (table 2):

TABLE 2

Dialysed plasma	N/1 NaCl	H_2O	N/40 $CaCl_2$	Thrombase (0·1 %)	Coagulation time
0·5	0·1	0·3	0	0·1	25
0·5	0·1	0·2	0·1	0·1	25
0·5	0·1	0·1	0·1	0·1	20
0·5	0·1	0	0·3	0·1	17

The results with dialysed plasma correspond to those observed with oxalated fowl plasma, viz. the time of coagulation by thrombase is reduced by calcium. The effect however is very small.

In this series of experiments it was observed that the coagulum formed by thrombase in the presence of calcium was much firmer than that produced in the absence of calcium. Possibly the properties of fibrin are determined to some extent by the presence of ionized calcium in the medium.

3. *The state of calcium in the plasma*

The discovery of Rona and Takahashi (1911, 1913) that calcium is present in serum in two forms, diffusible and non-diffusible, is now accepted. In the course of this work it was necessary to extend these observations to plasma.

(*a*) *The calcium content of dialysed fowl plasma.*

Dialysis against distilled water of fowl plasma contained in a collodion tube reduced the calcium content of the plasma from 10·8 mg. % to 3·0 mg. %. Thus 72 % of the total calcium was diffusible, whilst 28 % was non-diffusible. Precisely similar results were obtained with serum derived from this plasma. One ml. of the original plasma was coagulated by 0·1 ml. of a thrombokinase solution in 75 sec. After dialysis, the addition of thrombokinase did not produce coagulation in spite of the presence of 3 mg. % of calcium. Dialysis, however, takes a considerable time to come to completion, and although the fowl plasma did not coagulate during dialysis, and the precipitated fibrinogen, when redissolved by sodium chloride, produced normal coagulation with thrombase, it was nevertheless felt that some more rapid experimental process would give more reliable results.

(*b*) *The precipitation of calcium from fowl plasma by varying quantities of ammonium oxalate—coagulation by thrombokinase.*

Varying quantities of ammonium oxalate were added to fowl plasma. The quantities of calcium precipitated and remaining in solution were estimated. The results obtained are set out in table 3. For the sake of clearness the added oxalate is expressed in terms of calcium equivalents.

The results are expressed graphically in figure 1. The differentiation of the calcium into two forms is well brought out by the shape of the curve. Of the 11·8 % calcium contained in the original plasma, 9 mg. % were precipitated from the solution by a slight excess of ammonium oxalate, whereas the remaining 2·8 mg. were precipitated only by a large excess of the precipitant. The subsequent experiment shows that these two forms of calcium bear entirely different relations to the coagulation of fowl plasma by thrombokinase.

TABLE 3

Ca equivalent of added oxalate	Ca precipitated	Ca remaining in solution	Coagulation time with thrombokinase
0	0	11·8	40
12	3·6	8·2	50
18	5·8	6·0	95
24	8·6	3·2	335
30	9·5	2·3	No coagulation
36	9·5	2·3	,,
42	9·6	2·2	,,
48	9·7	2·1	,,
1000	11·8	0	,,

Quantities in mg. %; time in sec.

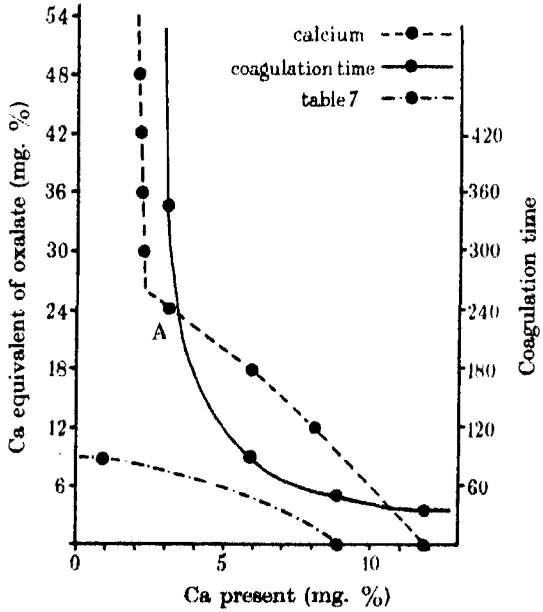

FIGURE 1

To each 1 ml. of the above fluid after removal, by centrifugalization, of the oxalate precipitate, 0·1 ml. of thrombokinase was added. The coagulation times are given in the fourth column of table 3. It will be seen that removal of half the total calcium reduced the coagulation time only from 40 sec. to 95 sec. By comparing these coagulation times with figure 1, it will be seen that the disappearance of the capacity to coagulate on the addition of the thrombokinase corresponds to the critical point *A*, i.e. the point at which all the calcium readily precipitated by oxalate (9 mg. % in this case) has been removed from the plasma. If this is so, the presence of 0·4 mg. % ionized calcium in the plasma suffices for the coagulation to be produced on the addition of thrombokinase in about 6 min. After the entire precipitation of this calcium, no coagulation took place within 1 hr. of the addition of thrombokinase.

These results confirm those stated above. It is therefore evident that when its calcium content is reduced to 3 mg. %, either by removal of the fraction readily precipitated by oxalate, or by dialysis, fowl plasma is no longer coagulable by thrombokinase.

(c) *The coagulation of diluted fowl plasma by thrombokinase.*

The form of the curve, relating the precipitation of the labile calcium with the amount of oxalate used, suggests that the added oxalate may associate itself with the proteins of the plasma. To this extent, therefore, the previous experiment may give erroneous figures concerning the amount of calcium required in plasma for coagulation to be induced by thrombokinase. The problem was therefore investigated by determining the degree to which plasma may be diluted with water and still retain the power to coagulate on the addition of thrombokinase. Globulin is not precipitated from fowl plasma on dilution with water. The results are given in table 4.

TABLE 4

Plasma	Ca content, mg. %	Coagulation time with thrombokinase
Original	10·8	45
Diluted 1:2	5·4	45
,, 1:4	2·7	80
,, 1:8	1·35	145
,, 1:16	0·67	445*
,, 1:32	0·33	Trace in 1 hr.

These figures indicate that a reduction of the total calcium to 0·67 mg. % and therefore of the ionized calcium to less than 0·3 mg. % (experiment *) still allows coagulation to be produced in 7 min. after the addition of thrombokinase. They confirm the suggestion that a soluble oxalate interferes with the coagulation of the plasma not only by precipitating the calcium but also by associating with the plasma protein.

4. *Calcium in the generation of thrombase from prothrombase*

(*a*) *The relation between the concentration of calcium and the time required for activation of a prothrombase solution.*

Thrombokinase and varying quantities of calcium chloride were added to a solution of prothrombase (0·1 %) and the times of complete activation determined by testing the thrombase activities of the resulting solution by means of oxalated ox plasma after varying periods of time. The figures given in table 5 show the relations between the calcium content of the fluid and the time required for complete activation.

TABLE 5

Ca content of thrombokinase-prothrombase solution	Time for complete activation
20 mg. %	15 min.
10 ,,	15 ,,
4 ,,	30 ,,
2 ,,	2 hr.
1 ,,	3 ,,
0 ,,	No activation

It is evident from these figures that in the presence of thrombokinase, prothrombase is converted to thrombase with the assistance of very small quantities of calcium (1 mg. %).

(*b*) *The precipitation of calcium from a prothrombase solution by means of a soluble oxalate.*

It has been shown above that to prevent the coagulation of plasma by thrombokinase soluble oxalate equivalent to three times the total calcium present must be added, and that even in the presence of this excess of oxalate, 3 mg. % of calcium is not precipitated. To determine whether this effect was in any way due to the association of prothrombase with calcium the following experiment was carried out.

A solution of prothrombase (0·1 %) and calcium chloride (calcium = 8·9 mg. %) was made up. The amounts of calcium thrown down from portions of this solution by varying quantities of ammonium oxalate were estimated and are set out in table 6.

It will be observed that the quantity of ammonium oxalate required to precipitate the calcium completely was twice the equivalent of the calcium present.

TABLE 6

Ca equivalent of added oxalate	Ca precipitated	Ca in solution
0	0	8·9
8·9	7·6	1·3
2 × 8·9	8·8	0·1
3 × 8·9	8·9	0

Figures indicate mg. %

(*c*) *The precipitation by oxalate of calcium from aqueous solution.*

Since the above figures showed that more than one equivalent of oxalate was required to precipitate completely the calcium from a prothrombase solution, an experiment was carried out to determine whether a similar fact held true for calcium dissolved in water. Varying quantities of ammonium oxalate were added to portions of a solution of calcium chloride in water of the same strength as that in the preceding experiment. Estimation of the calcium precipitated gave the results set out in table 7.

TABLE 7

Ca equivalent of added oxalate	Ca precipitated	Ca in solution
0	0	8·9
8·9	7·8	1·1
2 × 8·9	8·9	0

Figures indicate mg. %

It is evident that the precipitation of calcium from water by oxalate is precisely the same as the precipitation of calcium by oxalate from a prothrombase solution. Calcium therefore does not associate with the prothrombase so far as its capacity to be precipitated by a soluble oxalate is concerned.

(*d*) *Precipitation by oxalate of calcium from a prothrombase solution converted to thrombase.*

In this connexion it was of interest to determine whether in the activation of prothrombase to thrombase any change took place in the relations of calcium in so far as they could be determined by changes in response to a precipitating oxalate.

A portion of the prothrombase solution containing 8·9 mg. % of calcium, used in the experiment above, was converted to thrombase by the addition of thrombokinase. The figures in table 8 show the relation of the calcium to added oxalate in the active thrombase solution.

TABLE 8

Ca equivalent of added oxalate	Ca precipitated	Ca in solution
0	0	8·9
8·9	7·6	1·3
2 × 8·9	8·8	0·1
3 × 8·9	8·9	0

Figures indicate mg. %

From the above experimental results it is evident that the calcium takes up no special relation in regard to prothrombase or thrombase as determined by precipitation by soluble oxalate.

(*e*) *The spontaneous conversion of prothrombase to thrombase.*

It has been shown elsewhere (Mellanby 1933) that prothrombase precipitated by acid from a prothrombase solution, and after centrifugalization left moist at the bottom of a centrifuge tube, is converted into active thrombase within 24 hr. The results indicated that prothrombase in the presence of water or dilute acid changes into thrombase. To test this suggestion more accurately, 10 mg. of acetone-dried prothrombase was treated with a little dilute sodium carbonate to bring it to a neutral reaction and dissolved in 10 ml. of water. A chemical analysis of this prothrombase solution showed that no precipitable calcium salt was present. The activity of the prothrombase solution when converted into thrombase by thrombokinase and calcium was such that 0·1 ml. coagulated 1 ml. of oxalated ox plasma in 30 sec. The prothrombase was precipitated from 9 ml. of this solution by carbon dioxide and spun in a centrifuge tube. The supernatant fluid was poured off and the precipitate left in the bottom of the tube for 48 hr. At the end of that time it was dried by means of acetone and then redissolved in 9 ml. of water. The thrombase activity of this solution was such that 0·1 ml. coagulated 1 ml. of oxalated ox plasma in 25 sec., i.e. almost the same as that of the original prothrombase solution when activated.

Precisely similar results may be obtained when prothrombase powder is redissolved and precipitated by acetic acid or oxalic acid and left to activate. The results do not appear to depend upon the presence of acid, because a dried prothrombase preparation when moistened with water spontaneously activates, although at a slower rate than when precipitated from the solution by acid in a finely divided state. This is possibly due to the difficulty of wetting the dried powder.

The spontaneous conversion of prothrombase is a remarkable phenomenon, because not only does the prothrombase contain no detectable calcium, but also when tested on fowl plasma, it contains no detectable thrombokinase. Thus 1 ml. fowl plasma was not coagulated by 0·5 mg. of prothrombase which when submitted to the procedure described above was converted into a highly active thrombase preparation. Further it may be observed that thrombase differs in its properties from the prothrombase from which it spontaneously arose. Thus thrombase is soluble in water, and is not precipitated from the solution by the addition of acid.

Discussion

The relation of the calcium content of blood to coagulation time has been investigated by Stewart and Percival (1928*b*). They noted the (spontaneous) coagulation time of fresh samples of ox blood to which varying quantities of sodium oxalate had been added. To samples of serum from the same animal they added corresponding quantities of sodium oxalate and estimated the amount of calcium remaining in solution in each sample. From these experiments and from experiments with sodium citrate and sodium fluoride they concluded that calcium ions are not necessary for the coagulation of blood but that some complex non-diffusible calcium compound must be present to enable blood to clot. Given the advantage of a stable plasma it has been possible in the present experiments to make observations of a much more direct nature than these. The incoagulability by thrombokinase of fowl plasma from which all but about 3 mg. % of calcium has been removed, either by the addition of oxalate or by dialysis, strongly indicates that the calcium which is not readily precipitated by oxalate is identical with that which does not diffuse through a collodion membrane, and that this calcium plays no part in the process of coagulation.

It is generally stated that about 60 % of the serum calcium is diffusible, but various observers have noted that the exact proportion of calcium which will diffuse out of serum through a collodion membrane depends upon the conditions under which the process is carried out. Loeb (1924), for example, using human serum showed that the whole of the calcium is diffusible against 0·8 % NaCl, but that against distilled water 55–75 % only diffuses out at *p*H 7·4. The proportion of calcium which diffuses from stable fowl plasma against distilled water in 48 hr. (72 %) falls within these limits.

The fact that the calcium partition of fowl plasma is the same as that of fowl serum indicates that the processes of coagulation do not involve any redistribution of calcium in the medium.

Although Howell (1925) had prepared a thrombase free from phosphorus, Mills (1926) suggested that thrombase is a complex of prothrombase, calcium and cephalin. Ferguson (1937) came to the conclusion that whereas fresh thrombase is a prothrombase-cephalin-calcium compound which is inactivated by the removal of calcium, the thrombase later becomes stable and active even when deprived of calcium. However, the results here presented on the precipitability of calcium from water, prothrombase, thrombase and plasma, indicate that calcium does not combine with prothrombase or thrombase.

These experiments suggest that calcium must be allotted a far less commanding rôle than Howell claims for it. So far from corroborating the assertion that calcium alone may activate prothrombase and hence produce coagulation, they indicate that calcium is much less essential than is thrombokinase to the inception of the train of processes which ultimately result in the formation of fibrin from fibrinogen. Indeed it is probable that under certain circumstances prothrombase may be activated in the complete absence of calcium. If this is so then it is possible to meet the serious objection to the orthodox Morawitz-Fuld theory raised by Nolf (1938), who points out that oxalate plasma, as discovered by Howell, may be coagulated by the action of a number of substances, of which chloroform is the type. Nolf (1921) brought forward evidence to contradict the statement that this coagulant action of chloroform is due to the destruction of antithrombosin (antithrombase) which would in turn permit the action on fibrinogen of pre-formed thrombase. It is far more probable that the chloroform effect is comparable to the effect of trypsin, which in fowl plasma liberates thrombokinase (Mellanby and Pratt 1938). It is known (Mellanby 1935) that a very small quantity of calcium activates prothrombase and produces coagulation even in the presence of a tenfold excess of oxalate, provided the thrombokinase content is high enough. The normal effect of the presence of calcium in blood is merely to accelerate the rate of the reaction, but the reaction itself depends upon the availability of thrombokinase to the prothrombase, and it would appear that chloroform, like trypsin, makes thrombokinase available in the necessary manner.

If calcium can be relegated in this way to a subsidiary position in the scheme of blood coagulation it becomes possible to regard this activation of prothrombase to thrombase by thrombokinase and the activation of trypsinogen to trypsin by enterokinase as analogous processes. The fundamental difficulty in accepting such a point of view has lain in the apparent necessity for the presence of calcium in the former reaction, whereas in the latter it plays no part. Moreover, the activation of trypsinogen by enterokinase is not interfered with even by 1 % potassium oxalate. However, a consideration of the formation of thrombase from prothrombase isolated from the blood suggests that the difficulty may be largely fictitious. The rapid activation of prothrombase by the venom of *Lachesis atrox* (Mellanby 1938) and the slower spontaneous activation of prothrombase, both in the absence of calcium, emphasize the ability of thrombokinase to dispense with the adjuvant action of calcium in the formation of thrombase. Enterokinase and thrombokinase are called upon to operate under widely different conditions. It is clear that the activation of prothrombase to thrombase may be effected by thrombokinase alone when the conditions are adequate. Incidentally, it may be stated that thrombokinase and enterokinase are entirely different substances. A further striking similarity between the activation of prothrombase and that of trypsinogen is the form of their respective activation-curves. In each case, the velocity of the reaction is very slow at the beginning but a marked acceleration occurs as the reaction proceeds.

It is possible that the necessity for the presence of calcium salts when prothrombase is ordinarily activated in the blood is related to the association of fibrinogen with other electrolytes. It is known that the solution of fibrinogen in the presence of electrolytes is largely determined by the valencies and charges of the inorganic ions. In this case the calcium ions might be antagonistic to the sodium and potassium ions with respect to the soluble fibrinogen-prothrombase complex, and thus facilitate the action of thrombokinase on the prothrombase. Alternatively, or perhaps in addition, calcium may have some relation to the conditions under which thrombokinase is liberated in the blood.

SUMMARY

The calcium content of fowl serum is the same as that of plasma. Therefore calcium does not combine with fibrinogen in the formation of fibrin.

Calcium has no influence on the rate of coagulation of plasma by thrombase. Precipitation of calcium from fowl plasma shows that only 8–9 mg. % may be readily precipitated by a soluble oxalate. A great excess of oxalate is required to precipitate the remaining calcium. The coagulation of fowl plasma by thrombokinase is prevented when the calcium readily precipitated by oxalate is removed. A concentration of calcium ion of less

than 0·3 mg. % is required for the coagulation of fowl plasma by thrombokinase.

The rate of activation of prothrombase by thrombokinase is accelerated by the presence of calcium ions. The spontaneous activation of prothrombase in the presence of water, acetic acid and oxalic acid, shows that the calcium ion increases the rate of the reaction, but is not essential to the reaction.

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The fine structure of phloem fibres

I. Untreated and swollen hemp

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[Plates 14, 15]

Introduction

During an anatomical study of the jute and hemp plants carried out by one of us (B. C. K.), the opportunity was taken of reinvestigating in these plants the structure of the walls of the fibres, using the term "fibre" in its botanical sense. The so-called "bast" fibres are of particular interest in wall studies since it was apparently on the basis of their investigation that the "crossed fibrillar" conception was first introduced. Thus we find Reimers (1922) stating that in a number of plants, including hemp, the walls of these fibres are composed of two or more layers differing considerably in cellulose-chain direction. Since that time numerous other statements of this kind have been made for different types of cell (Freudenberg and Dürr 1932; Ritter 1930; Ritter and Chidester 1928; Bailey and Kerr 1935; v. Iterson 1937; Wuhrmann-Meyer 1939). It seems rather a common feature of the evidence quoted in support of such a structure that it is derived from material swollen considerably either in acid or alkali. Certainly the optical properties of the wall have been used in support (Freudenberg and Dürr 1932; Bailey and Kerr 1935) but the implications of such work have already been discussed elsewhere (Preston 1939*a*). We are here concerned chiefly with the swelling technique. While it is not to be suggested that treatment with swelling reagents totally invalidates all observations of this type, it is quite clear that in some cases at least observation of swollen material can give an entirely erroneous conception of the wall in its natural condition. This is perhaps particularly clear in the case of jute, for which Osborne (1935, quoted also by Barker 1938) refers to the fibres as being composed of a series of chains lying at a considerable angle to the longitudinal axis of the cell, in spite of contrary X-ray evidence.

In general the swelling of the plant cell wall must be a complex process, causing considerable changes in wall structure, particularly in those cells which fail to swell uniformly. The multiplicity alone of the phenomena revealed—concentric lamellae (Balls 1919; Bailey and Kerr 1935; Kerr 1937; Farr 1938), fibrils (Balls 1922), crossed fibrils (Reimers 1922; Ritter 1930; Bailey and Vestal 1937), "ballooning" (Lutdke 1928, 1932; Bailey and Kerr 1935; v. Iterson 1927; Farr 1938)—would indicate the complexity of the problems concerned when the attempt is made to interpret the intact wall on the basis of observations of swollen material. It is the aim of the present paper to show that treatment, even with weak swelling agents, produces sufficient distortion to render almost useless application of the swelling technique in the elucidation of the fine structure of the plant cell wall.

The results to be presented here refer exclusively to the hemp fibre—jute will be considered in a later communication. The fibres appear to occur only in phloem tissue and are to be considered as primary or secondary according to whether they develop from procambium or from cambium. With both classes of fibre an attempt is made to establish a connexion between the various swelling phenomena mentioned above and to contrast these with the structure of the intact wall as derived by a variety of methods. It has become quite clear that the presence of substances other than cellulose has a profound influence on the results of the swelling process. Observation of swollen material can be and often is deceptive, and the structures thus revealed cannot legitimately be taken as typical of the intact wall.

MATERIALS AND METHODS

Plants of *Cannabis Sativa* were obtained from Reading in October 1937, through the courtesy of Messrs Sutton and Sons, and in the 1938 season plants were grown in the open in the University gardens. The fibres were extracted, both from growing and from adult stems, by two methods—maceration in 5 % chromic acid, and retting in water alone. In the second method, whole plants were placed in vats filled with water where they remained until the necessary stage of maceration was reached. By this process, the whole group of primary and secondary fibres (the commercial "fibre") could be obtained with ease, but it proved difficult later to separate the two classes of material. This method was therefore replaced by one in which pieces of internode were placed in large photographic dishes containing tap water and the process of retting watched each day. Complete retting occurred at the end of one month, but after a period of three weeks it was found possible to separate the outer, primary, fibres after a little manipulation. These were placed in water in small specimen tubes and the retting process continued until complete separation took place. The secondary fibres were later separated from the wood, and after complete maceration the two kinds of fibres were stored in 30 % alcohol in separate tubes. Maceration in chromic acid followed a somewhat similar course, the acid being changed every day until complete maceration was observed.

For the bulk of the work it proved necessary to obtain single fibres. No difficulty was encountered in dealing with the chromic macerated material, though the water-retted samples gave rather more trouble except when the retting process was more prolonged than usual. Fine steel or glass needles were used in the separation of the fibres.

Since the breadth of the fibres varied considerably along their length, special precautions had to be taken whenever it became necessary to measure the fibre diameter, particularly when changes in diameter were to be determined. For this purpose, individual fibres were air dried and mounted on a slide by attaching the two ends with "Durofix". The slide had, previously etched on it, fine lines parallel to its shorter edge, and measurement of diameter was made only at the points where the fibre crossed these lines. Any rotation of the fibre could generally be detected with the aid of drawings marking individual peculiarities. For the purpose of applying pressure, very thick cover-glasses were used, and pressure applied either in a vice for general pressure, or with the aid of a thin wooden rod with a rounded end for localized pressure.

STRUCTURAL FEATURES OF THE UNTREATED WALL

The point at issue in the present paper centres around the question of the existence in the intact wall of the hemp fibre of layers differing in chain direction, such as swelling phenomena would seem to indicate. Several methods are now available for the investigation of the structure of the wall. Thus the X-ray diagram gives information directly as to the orientation of the cellulose chains composing the wall, and in the X-ray method we have, in fact, the only reliable means of determining this property. Certainly the criticism may be made that in a wall apparently built up of only one set of chains there may exist a layer, differing in chain direction and yet so thin that its diffraction spots fail to be recorded. It has, however, been shown recently (Preston and Allsopp 1939) that even in such weakly fibrous material as wood, layers could be detected at least

$\frac{1}{20}$th of the thickness of that actually giving a strong X-ray diagram. This is of particular importance since those layers in a wall, whose optical properties are so different from those of the rest of the wall as to have led to the conception of a different chain direction (e.g. Bailey and Kerr 1935), are generally much thicker than would be represented by this figure. Again, striations are often visible on the wall and these have been shown in some cases (*Valonia*, Preston and Astbury 1937; *Chaetomorpha*, Nicolai and Frey-Wyssling 1938; *Cladophora*, Preston and Astbury 1939) to correspond in direction to the cellulose chains in the wall. It is not, however, always easy to distinguish between striations proper and other wall markings, and there are already in the literature a few cases in which apparent striations are *perpendicular* to cellulose chain direction (Farr and Sisson 1939). Of perhaps rather less wide applicability is the determination of the major extinction position, for this direction may be taken to correspond to the cellulose chain direction only if it is known that only one set of chains is present. A coincidence of striation direction and major extinction position would argue strongly in favour of the existence of only one set of chains. Finally, determination of the optical properties of the wall in section, though favoured by some authors, is clearly of doubtful value unless supported by other evidence (Preston 1939*a*, *c*). Such optical properties are controlled by so many factors that it is not at present feasible to attempt definitely to correlate them with the direction of cellulose chains alone.

X-ray diagrams of "bast" fibres have been published extensively during the evolution of modern ideas on the structure of cellulose. These diagrams (chiefly of ramie fibres, but occasionally of hemp (Astbury, Preston and Norman 1935)) show no sign of more than a single orientation of cellulose chains. In the present investigation single fibres have been examined from this point of view, both before and after mercerization, and no trace of a second orientation has been detected even after the fibres have been subjected to rather severe mechanical treatment. The cellulose chains appear to lie almost longitudinally (figures 5, 6, plate 14) and any wall layer with a different orientation must be tenuous in the extreme.

Microscopical observations of macerated fibres, whether water retted or treated with 5 % chromic acid, reveals the presence of striations which are never far removed from the longitudinal (table 1); these are the only true striations visible in the unswollen wall. Other markings are often noticeable, but none of these have any relation to striations proper. Thus "slip planes" can readily be recognized on account of their optical properties, as so often described for fibres. The occasional appearance of markings,

TABLE 1

	Thickened fibres				Unthickened fibres.	
	Primary		Secondary		Secondary	
Fibre	θ	θ'	θ	θ'	θ	θ'
1	−3·0	−3·0	+4·0	+4·5	+4·5	+3·0
2	−0·5	0·0	+4·0	+4·0	−3·5	−1·5
3	0·0	−1·0	+4·5	+3·5	−3·5	−2·0
4	+1·5	+0·5	0·0	−1·0	−4·0	−4·5
5	+3·0	+2·5	+5·0	+4·0	+1·5	−1·5
6	+6·0	+3·0	+3·5	+2·5	0·0	+1·0
7	−2·0	−2·0	−0·5	−0·5	−3·5	−3·0
8	−4·5	−3·5	0·0	0·0	−1·0	+1·0
9	−6·0	−1·5	+3·5	+2·5	−1·0	−1·0
10	−5·0	−5·0	−1·5	−0·5	−5·0	−5·5
11	−2·5	−1·0	−1·0	−1·5	+0·5	−2·0
12	0·0	0·0	−2·0	−1·0	−1·0	−1·0
13	−4·5	−4·0	−4·0	−1·5	−6·0	−5·0
14	+1·0	−1·5	−1·5	−1·5	+2·5	+1·0
15	−1·0	+1·0	−3·5	−2·5	—	—
16	−6·0	+2·5	+2·0	+1·5	—	—
17	−5·0	−1·0	+1·0	+1·0	—	—
18	−3·0	−3·0	−2·5	−2·5	—	—
19	−2·5	−3·0	+2·0	+2·0	—	—
20	+4·0	+2·5	+4·0	+4·0	—	—
21	+1·5	+1·0	+0·5	+0·5	—	—
22	—	—	−1·5	−1·0	—	—
23	—	—	−5·5	−5·0	—	—
24	—	—	−0·5	+1·0	—	—
25	—	—	+3·0	−2·0	—	—
26	—	—	+3·0	+2·5	—	—
27	—	—	−0·5	0·0	—	—
28	—	—	0·0	0·0	—	—
29	—	—	0·0	0·0	—	—
30	—	—	−5·0	−6·0	—	—
Av.	3·0 ± 0·4	2·1 ± 0·3	2·3 × 0·3	2·0 ± 0·3	2·7 ± 0·5	2·4 ± 0·4

θ = The inclination of the major extinction position to the fibre axis (degrees).
θ' = The inclination of the striation direction to the fibre axis (degrees).
Negative signs imply a right-handed spiral, and positive a left-handed.

frequently transverse but often so arranged as to enclose a six-sided area, may be attributed to the presence of parenchyma in contact with the fibre before maceration (figure 1). They possibly represent lines of thickening in the primary wall of the fibre at its junction with two adjacent parenchyma cells. Again, the faintly visible transverse markings which may be seen on the outer layers of the wall, even when these latter structures are absent, appear to be folds rather than striations. The considerable amount of evidence in the literature concerning the relation of striation direction to cellulose chain orientation would again suggest that the hemp fibre is largely composed of chains running in a steep spiral. This is supported and extended by observations of single walls under the polarizing microscope.

The major extinction position (m.e.p.) of the wall is always inclined at a small angle to the longitudinal axis of the fibre. Some difficulty is experienced in measuring its precise direction, since the thickness of the wall in comparison with the lumen makes it impossible to obtain any large area of single wall for observation. Nevertheless the degree of accuracy is sufficient to allow comparison of major extinction position and striation direction. The degree to which these correspond (table 1) is very striking, particularly in view of the above difficulty and of the fact that the latter directions are often taken from areas adjacent to those used for the former measurement. The striation direction is often coincident with the m.e.p. and the two are never widely separated. Such agreement could hardly be expected of a wall an appreciable part of whose thickness is built up of chains lying at an angle different from that in the rest of the wall. It seems reasonable to suppose that such a layer is absent. Such observation of swollen material as suggests that the cellulose chains in an outer layer in the wall are oriented more nearly transversely will be examined in a later section. It is sufficient here to point out that in retted fibres soaked in water for many days this outer layer can be dissected off. Fibres under these conditions show a series of boat-shaped cracks (figure 2) with their major axis lying longitudinally to the fibre, and these are visible both in the bulk of the wall and in the thin, dissected, outer layer. The m.e.p. of these dissected fragments also lies longitudinally, as does that of the rest of the fibre wall, and this, taken in conjunction with the fact that the

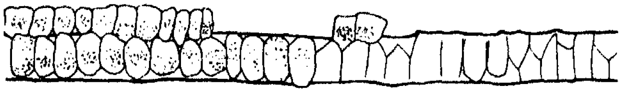

FIGURE 1. Unthickened primary fibre with parenchyma attached and showing marks resembling the pattern of parenchyma cells. (× 130.)

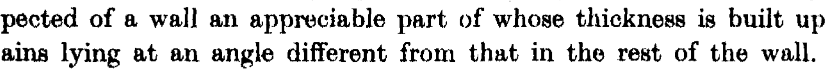

FIGURE 2. Retted primary fibre showing longitudinal boat-shaped cracks piercing the whole wall thickness. (× 130.)

wall consists in the main of cellulose chains lying parallel to the length of the fibre, leads inevitably to the conclusion that the outer layer itself is composed of chains lying in a similar orientation. Further support for such a conclusion is contained in the fact that the m.e.p. of slightly thickened fibres is not significantly different from that of fully thickened (table 1). It may be pointed out that this tenuous weakly birefringent outer layer is strongly reminiscent of a primary wall, though no definite pronouncement can be made on this point. Certainly preparations of primary wall material from young internodes show similar perforations which, in older, still unthickened fibres, are similarly oriented, though in young fibres they lie along a fairly flat spiral. Here, then, would appear to be one more case in which the cellulose chains in the primary wall are not always oriented transversely.

In spite, however, of this apparent homogeneity in the cellulose chain direction the fibre wall is decidedly heterogeneous when examined in thin cross-section under the polarizing microscope. The bulk of the wall is isotropic or nearly so (figure 7, plate 14), as is necessitated by the presence of cellulose chains oriented nearly longitudinally; but an outer lamella is also present which is bright and therefore birefringent when observed between crossed Nicols. The path difference in these lamellae, in 10μ sections, varies from 200 mμ in secondary to 160 mμ in primary fibres. It is not quite clear what factors are involved in this sudden change in optical properties. Certainly the thickness of the lamella, in view of the evidence presented above, is too great for the whole of it to consist of chains of cellulose lying less steeply inclined than those already discussed. Similar observations on another type of cell (Preston 1939*a*) have been shown to receive a possible explanation in terms of an angular dispersion of the cellulose chains, but it cannot as yet be determined whether the same considerations apply here.

STAINING REACTIONS OF THE WALL

In a later section it is proposed to deal with the swelling reactions of the wall in chromic-macerated and water-retted fibres, and to this end it is profitable for a moment to consider the relation of cellulose to the other wall constituents in the various wall layers. While the observations to be discussed here refer chiefly to staining methods, sufficient use has been made of specific solvents to ensure that the technique yields results of qualitative and roughly quantitative importance. The presence of cellulose was detected by the usual treatment with iodine and sulphuric acid, and with chlor-zinc-iodine. Lignin was tested for by its reaction with phloroglucin and hydrochloric acid and with aniline chloride, supported by extraction with alternate treatments with Eau de Javelle and hot sodium sulphite. Finally, methylene blue was used as a staining reagent for substances which may be classed as "pectin".

In transverse section of the stem the fibres never give a positive staining reaction for cellulose; primary fibres invariably show a bluish brown coloration much more intense than the reddish brown of secondary fibres. This fact is in harmony with the observation of other workers on lignified tissues and would suggest a higher degree of lignification in secondary than in primary fibres. This suggestion is supported by the staining reactions for lignin. In phloroglucin and hydrochloric acid, primary fibres remain unstained with the exception of a faint reddish coloration in the middle lamella. In secondary fibres, however, all layers lying outside of, and including, the first layer of the secondary wall are deeply stained. The primary wall is unusually thick in the corners of a cell and here the colour is more intense. The remainder of the secondary wall is faintly stained.

After retting in water, on the other hand, the wall develops a deep blue or bluish violet in the iodine reagents mentioned. This suggests immediately that some at least of the lignin has now been removed. In harmony with this observation, it is found that staining tests for lignin are negative in primary fibres, except for a faint coloration in the primary wall region. In secondary fibres the bulk of the wall is still faintly stained and the primary wall particularly is still highly lignified.

After maceration in chromic acid, the test for lignin is universally negative and now, as expected, the wall develops a deep blue colour with iodine and sulphuric acid.

The presence of "pectin" as defined above may be detected in primary walls and middle lamella of secondary fibres only. The secondary walls of these fibres, and the whole wall of primary fibres, nowhere show the blue staining reaction with methylene blue, and the latter, in fact, often appear faintly pink. After retting in water the whole of the pectin has apparently been extracted and the same applies to treatment with 5% chromic acid.

Although colour reactions are unsatisfactory in some respects, it seems that the distribution of cellulose, lignin and pectin may roughly be outlined as follows. Both primary and secondary fibres are composed of cellulose, lignin, pectin, and other substances for which no specific tests have been made. In primary fibres the lignin is largely confined to the middle lamella and primary wall, while in secondary fibres the whole of the wall shows distinct evidence of lignification. Failure of the test for cellulose until after the lignin has been removed is an observation common to many lignified tissues, and leads to the conception of lignin as ensheathing the micelles, or micelle aggregates, of cellulose (Freudenberg and Dürr 1932), a conception which is supported by other evidence (Astbury *et al.* 1935; Preston and Allsopp 1939). Pectin, as here defined, seems to be absent except in the middle lamella and the primary wall of secondary fibres.

When the fibres are retted in water considerable changes occur both in lignin and in pectin content. As judged by the colour reactions, pectin disappears completely while lignin diminishes considerably in amount, particularly in secondary walls. This removal of incrusting substances proceeds still further in chromic acid, where colour reactions for constituents other than cellulose fail entirely.

THE EFFECT OF SWELLING AGENTS

It seems reasonable to suggest that the precise nature of the swelling process depends not only on the configuration of the cellulose complex itself, but also on the distribution of the incrusting substances. This suggestion is fully borne out by the results of the swelling in sulphuric acid and in caustic soda of fibres in their intact condition, after retting in water, and after treatment with chromic acid. As seen in transverse section of the stem, both primary and secondary fibres fail to swell in Schweizer's reagent. In concentrated sulphuric acid, however, they swell considerably though in many cases, particularly among secondary fibres, they do not dissolve even after prolonged treatment. Solutions of caustic soda in strengths of 10 % or more cause appreciable swelling in both cases. After retting in water both primary and secondary fibres swell and dissolve immediately in strong sulphuric acid, with the exception of an outer layer and occasionally of the layer next the lumen; these dissolve more slowly, the outer layer being the more resistant. Appreciable swelling occurs on immersion in acid down to 55 % concentration; this will, however, be discussed in detail later on. Secondary fibres, which are definitely more lignified than primary fibres, invariably show a stronger resistance to sulphuric acid. In caustic soda, careful measurement shows the fibres to be more swollen than those treated in the intact condition, and here again the fibres in the secondary groups are the more resistant. After maceration in 5 % chromic acid all fibres swell and dissolve completely in sulphuric acid of a strength of 60 % or more, outer layers being rather more resistant. In strengths below 60 % the outer layer and the layer lining the cell lumen fail to dissolve even after prolonged immersion.

In all these cases of swelling, broadening of the fibre is always accompanied by some longitudinal contraction. Special cases of this will be discussed later. It may be pointed out, however, that differential swelling of layers, such as has been discussed above, will lead to differential shrinkage. This undoubtedly causes many features to develop during swelling which have no existence in the intact fibre.

Resistance to swelling thus seems largely attributable to lignification, since swelling becomes more pronounced as lignin is removed from a layer, and since the outer, more strongly lignified, layer is always the most resistant. At the same time the fact is rather suggestive that an inner layer, which is not appreciably more lignified than the rest of the secondary wall, fails to swell. It points either to the inadequacy of the lignin test or, more probably, to a specific effect upon swelling of the precise condition of the cellulose itself. Resistance to swelling is not, however, to be attributed to a difference in cellulose chain direction, for the outer layer only is optically different from the rest of the wall.

As regards the effect of swelling on wall structure, material has been examined in detail over a rather wide range of swelling conditions, and the multiplicity of phenomena observable justifies very fully the scepticism already expressed as to their value in wall studies. Even long-continued immersion in water of water-retted fibres causes pronounced changes in their appearance. The transverse markings already mentioned as an occasional feature of the intact wall become much more numerous in the outer wall layers and also begin to appear in the innermost layer. Treatment for 1 hr. with 10% caustic soda causes these markings to become so much more pronounced that they may clearly be recognized as folds (figure 8, plate 14). Under the polarizing microscope each fold appears as two transverse bands, almost isotropic, with a narrow band running between them possessing the same optical properties as the unfolded wall. This is exactly what we should expect from such a fold. More prolonged treatment has no further effect beyond a slow general swelling. On the other hand, fibres macerated in chromic acid and treated for 19 hr. with 10% caustic soda develop a series of transverse cracks (figure 3*a*). These are particularly numerous if the fibre is allowed to dry for 15 min. before the application of the caustic soda, and are more readily observable in secondary than in primary fibres. They recall at once the "chemical sectioning" of ramie fibres observed by several investigators (e.g. Velaney and Searle 1930). The cracks are initiated as transversely oriented, boat-shaped crevasses in the wall (figure 3*a*), which rapidly develop through the central layers of the wall as these contract in length.

The cellulose chains in the wall now run parallel to the distorted wall surface (figure 3*b*).

Primary fibres macerated in 5 % chromic acid and immersed in 10 % caustic soda occasionally present a strikingly different appearance, which may be observed more abundantly among secondary fibres in 50 % sulphuric acid. Since this phenomenon was first observed, careful observation of the initial swelling process under these conditions has revealed that, particularly in sulphuric acid, an outer layer of the secondary wall together with the primary wall often separates from the fibre, and the present observations are to be explained in terms of this chemical dissection (figure 4). The outer layer first cracks along a steeply spiral line so that as the internal layers of the fibres swell they protrude along this fissure and push apart the turns of the outer spiral causing it to assume a progressively greater inclination to the fibre axis. Thus we have the appearance of what may be termed "spiral ballooning" (figure 9, plate 14;

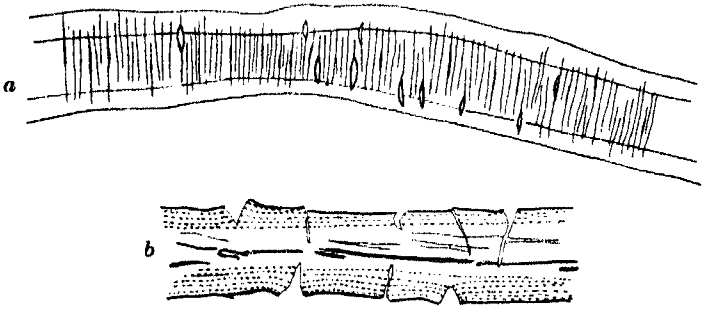

FIGURE 3. Chromic macerated fibres after immersion in 10 % NaOH solution for 19 hr. or longer. *a*, the initial stage of swelling showing the development of transverse markings and fissures. *b*, the transverse fissure well-marked and causing marked distortion of the direction of cellulose chains (dotted lines). (× 130.)

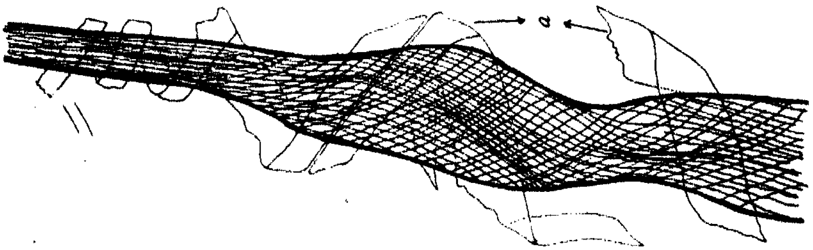

FIGURE 4. Water-retted primary fibre soaked in 10 % NaOH. The primary wall (*a*) has been teased off with needles and the treatment has caused the wall to break up into fibrils which are less steeply inclined in the more swollen regions. (× 130.)

figure 11, plate 15). This phase is, however, passed through rather rapidly. Locally pronounced swelling causes localized excessive flattening of the spiral band, which then apparently slides along the balloons, collects at a neighbouring point of excessive constriction and leads eventually to the typical "ballooning" so frequently described (figure 11, plate 15). It is quite clear that this "ballooning" in hemp arises as a direct consequence of the presence of an outer, relatively non-swelling layer which cracks and is distorted by underlying layers with greater swelling power. The initial steps of "ballooning" here differ apparently from those in the cotton hair, where the cracks are reported to develop transversely and longitudinally (Farr 1938). The transverse arrangement of the fibrils at points of constriction is clearly a consequence of swelling; the first fissures in this outer layer are never far removed from the longitudinal. Certainly their presence is not to be taken as evidence for a similar orientation in the intact wall as implied by some workers.

This line of argument is further supported by the fact that more prolonged immersion in sulphuric acid (one hour or more) generally causes swelling sufficiently great to remove this outer constricting band; the constrictions now swell to the same extent as the neighbouring "balloons", and the fibre appears uniform in diameter (figure 12, plate 15). It is at this stage that a point arises fundamental to the applicability of swelling technique. Whereas in the intact fibre the cellulose chains, of internal layers at least, are oriented almost longitudinally, the wall is now often seen to have developed fibrils lying in comparatively flat spirals. Much of this flattening is undoubtedly connected with lack of care in handling the swollen material. If the cover-glass is replaced gently after the addition of the caustic soda or sulphuric acid only a slight change in fibrillar direction is observable (table 2). This is to be expected since the initial inclination to the vertical is so small. Such changes are nevertheless real and become more marked with increased swelling (table 2). Occasionally they are accompanied by a pronounced flattening of the fibrillar spiral in the outer layers of the wall (table 2, fibres 6–9), a point of some significance in view of the presence of an internal non-swelling layer. The slightest carelessness in applying the cover-glass, however, leads to a most striking change in the appearance of the fibre. The smallest pressure causes a pronounced increase in the inclination of the fibrils and they become more clearly visible as a series of continuous spiral threads (figure 10, plate 15).* With increased pressure, the inclination increases progressively (table 3) and the wall now has the appearance figured by Osborne (1935), for jute, as typical of the fibre. It is quite clear that the fibrillar orientation of such fibres is a direct consequence of dimension changes in the fibre and is readily understandable in terms of the geometry of the spiral. A similar flattening of the fibrillar spiral may be observed on localized compression of either

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* The X-ray diagram of single fibres in this condition shows conclusively that the directions of cellulose chains and of fibrils still correspond. Unfortunately the photographs are not sufficiently intense for reproduction even after 100 hr. exposure.

TABLE 2. THE CHANGE IN DIAMETER AND STRIATION DIRECTION OF SECONDARY FIBRES ON TRANSFERRING TO PROGRESSIVELY GREATER STRENGTHS OF CAUSTIC SODA SOLUTION

	Diameter (μ)					Inclination (degrees)				
Fibre	Water	10 %	20 %	30 %	40 %	Water	10 %	20 %	30 %	40 %
1	31·2	39·7	42·0	43·3	46·6	0·3	0·3	1·2	2·9	3·2
2	31·0	43·1	46·2	45·0	50·7	0·2	0·2	0·5	0·5	3·0
3	28·3	33·0	36·5	37·2	40·9	0·2	0·3	0·9	1·0	1·4
4	31·3	57·0	60·5	63·0	53·4	0·2	1·9	2·5	3·8	2·5
5	42·0	57·7	65·7	64·5	64·0	0·5	2·4	1·2	—	1·4
6	40·5	—	51·7	—	—	1·2	—	0·2 (39·6)*	—	—
7	27·0	—	38·2	—	—	1·0	—	0·8 (48·0)*	—	—
8	31·5	—	49·5	—	—	0·5	—	2·6 (7·6)*	—	—
9	29·2	—	47·2	—	—	1·0	—	1·0 (10·6)*	—	—

* Figures in brackets refer to the outermost layers of the wall.

TABLE 3. PRIMARY FIBRES IMMERSED OVERNIGHT IN 10 % CAUSTIC SODA. DIAMETER IN μ; INCLINATION IN DEGREES

	Cover-glass replaced gently		Cover-glass dropped		Gentle pressure		Heavy pressure	
Fibre	Diam.	Inclination	Diam.	Inclination	Diam.	Inclination	Diam.	Inclination
1	55	*ca.* 0	72	16	72	19·5	73	27
2	31	*ca.* 0	43	8·5	49·5	16·0	72	22·0
3	36	*ca.* 0	58	12·5	63	14·0	81	19·0
4	36	2·0	90	16·0	90	16·0	103	22·0
5	36	*ca.* 0	61	20·0	66	21·0	67	19·0
6	35	1·0	57	17·5	59	17·0	112	33·0
7	54	6·0	84	19·0	86	20·0	153	35·0
8	37	*ca.* 0	59	18·5	59	23·0	77	30·0
9	45	1·5	64	19·0	65	19·0	95	29·0
10	77	*ca.* 0	94	25·5	124	27·0	166	37·0
11	54	3	81	17·5	90	23·0	101	29·0

swollen or untreated fibres (figure 10, plate 15). As a further point it may be mentioned that consideration of the geometry of the case demands that unless the longitudinal swelling of the fibrils is marked a considerable shortening of the fibre should occur during swelling and compression. In agreement with the results of Lutdke and others such a shortening is, in fact, observed even during swelling alone (table 4).

TABLE 4. CUT PIECES OF FIBRE TREATED WITH 5 % H_2SO_4

	Before treatment with H_2SO_4			After treatment with H_2SO_4			After pressure		
	Length μ	Breadth μ	Angle of striation	Length μ	Breadth μ	Angle of striation	Length μ	Breadth μ	Angle of striation
1	1080	42	*ca.* 0	1057·5	45	*ca.* 0	997·5	140–200	17·5–24°
2	2235	34	,,	2190	37	,,	2085	97–221	7 –21°
3	2025	35	,,	2010	38	,,	1935	108–288	12 –36°
4	3087	48	,,	3062·5	52	,,	2625	346–450	30 –50°
5	2200	32	,,	2150	38	6°	1937·5	150–312	31·5–40°
6	1850	44	,,	1787·5	48	*ca.* 0	1662·5	187·5	17 –21°
7	862·5	32	,,	843·5	64	2·5°	781·5	155–462	22 –40°
8	1100	30	,,	1075	34	*ca.* 0	1050	68–154	5 –17°
9	875	30	,,	800	36	,,	775	112	15°
10	1685	42	,,	1685	47	,,	1635	102	12°

DISCUSSION AND CONCLUSION

Several lines of investigation thus lead to the suggestion that the phloem fibres of hemp are built up of cellulose chains running in a steep spiral, with possible occasional exceptions. Such observations as have been made on fibre development, though by no means conclusive, suggest that during cell elongation the chains in the primary wall move from a more transverse position to a direction comparable with that of the chains in the secondary layer later deposited upon it. On this view the presence of an outer layer with a path difference in cross-section higher than that of the remainder of the wall is not easy to understand. In view of the many factors which may affect the double refraction of a colloid like cellulose, it seems rather unsafe to assume on such evidence alone that an outer layer is universally present with an almost transverse orientation. Perhaps it may be significant that the path difference of this layer is higher in secondary fibres than it is in primary fibres which are the less strongly lignified, though according to Kanamaru (1934) high lignin content implies low birefringence. Of further interest is the observation of this worker that the double refraction of hemp fibres in longitudinal view corresponds closely to that of cellulose micelles, particularly after incrusting substances have been removed. Since he uses the Becke line method, his figures would seem to refer to the outer layer of the wall in which, therefore, the cellulose chains must not be very far removed from the longitudinal direction. Kanamaru, however, apparently used commercial fibres, and in view of the relative ease with which the primary wall may be stripped from the fibres (it may be observed coming away from the secondary wall even after prolonged immersion in water alone) it is not clear whether or not the primary layer was present in his material.

A strong argument against the change of cellulose chain orientation from layer to layer is the fact that, on swelling, the outer layers of the wall crack first along a very steep spiral, which subsequently becomes flattened only by the swelling of layers to the inside; and that whenever this outer layer is dissected off both fissures and the major extinction position are almost longitudinal. In this case, as in the conifer tracheid (Preston 1934) and unlike the sporangiophore of Phycomyces (Oort and Roelefson 1932; Castle 1936), both fissures and m.e.p. lie in the same direction.

The swelling of the wall is clearly related to the different swelling properties of the various wall layers present, and is best understood in terms of their chemical differences. There are universally present outer and inner layers whose swelling is much less pronounced than that of the thicker layer between them. This difference is undoubtedly connected, to some extent, with the nature and amount of the incrusting substances; for as these are removed the outer layers become comparable in swelling reaction to the central layer. On the other hand the inner layer is apparently no more intensely lignified than the swellable central layer. Taken in conjunction with the fact that even after delignification the outer layer and inner layer are still resistant to swelling and that unthickened fibres fail to swell almost entirely, this would seem to infer a configuration of the cellulose composing them different in some respects from that of the central layer. Such difference cannot be changes in chain direction for the inner layer has optical properties identical with those of the central layer. Thus in a single fibre we have further evidence in support of the attempt of Sisson (1936), in terms of the theory of crystal structure proposed by Zwicky, to effect a compromise between the discrete micelle of Nageli and the existence, in cellulose, of a continuous structure demanded by some properties of fibres. Here we have the swelling properties of different layers of the wall as widely different as those between different types of fibre, although the chemical composition of the layers cannot be very different. The arrangement and association of the "micelles" must differ in the different layers.

It has been shown that the lateral swelling involves longitudinal shrinking and it is in these terms that the appearance of transverse folds and cracks is to be explained. On slight swelling the central layers alone contract and cause both inner and outer layers to be thrown into folds, the number of folds increasing with increased swelling. If the fibre has been treated previously with chromic acid and the swelling is more intense, the inner layer apparently ceases to be thrown up into folds and a longitudinal tension is set up in the central layer, leading to a series of transverse cracks which are always wider on the outer side, i.e. farthest from the non-swelling inner layer. Clearly treatment with chromic acid has removed the resistance of the outer layer to swelling by removal of lignin and perhaps also by a simultaneous change in the relation of the cellulose crystallites. The regular occurrence of such transverse cracks on treatment of fibres whose primary wall has been removed lends further support to this view. The "ballooning" phase of swelling is also clearly to be attributed to a failure of the outer layer to keep pace with the internal swelling. If this latter be rapid, then the outer layer becomes cracked along a spiral line, giving the appearance of spiral ballooning which eventually leads to the more typical form as already described.

Finally we have the effect of swelling on cellulose chain direction. Mercerization inevitably makes a fibre more responsive to treatment, particularly of a mechanical kind. The slightest carelessness in manipulating the swollen fibre causes profound changes in chain direction, and the presence of an inner resistant layer causes these changes to be often more marked in outer regions of the swelling layer. The effects of swelling alone (omitting the effect of handling and of "ballooning") are here rather small, but it would seem that they may be much more pronounced in cells whose cellulose chains are less steeply inclined. It is quite clear that the structure of the swollen wall bears no resemblance to that of the intact wall, and no picture of wall structure based only on material treated with swelling agents can possibly be accepted.

In conclusion, although such observations as have been made on development of the primary wall hardly merit any lengthy discussion, it may perhaps be emphasized that they do definitely indicate a more or less transverse orientation of cellulose chains which become longitudinal in the adult fibre through the agency of growth. The conception of a primary wall with longitudinally oriented chains at any stage of its existence seems to be at variance with the bulk of the literature (Bonner 1936; van Iterson 1937; Castle 1936;, Wuhrmann-Meyer 1939), and the idea that cellulose chains may be changed in orientation during growth seems to be losing favour. It is unfortunate that in the bulk of such work it is not clear that sufficient care has been taken in the determination of optical properties and in their relation to the geometrical features of cell enlargement. Optical determination of the properties of a cell wall are clearly quite inadequate unless they are carried out on *single* walls. In hemp we have the clearest evidence that the bulk of the primary wall is composed of longitudinally directed cellulose chains. It cannot be denied, however, that in this tenuous wall there may occur layers (perhaps in a molecular sense) whose chain direction is different—in fact considerations of growth in terms of wall extension would appear to necessitate a steepening of the chain direction in passing from an outer to an inner layer here, particularly if wall deposition goes on by apposition rather than by intussusception.

Description of Plates

Plate 14

Figures 5, 6. X-ray diagrams of hemp fibres after water retting (figure 5) and after treatment with 10% caustic soda (figure 6). 100 hr. exposure to CuK_α radiation from a Philips Metallix tube at 40,000 V and 25 mA.

Figure 5. Two fibres arranged almost parallel (fibres parallel to the long edge of the plate). The equatorial spots are clearly double, so that very small differences in cellulose chain directions can be detected by this X-ray method. Single fibres give single photographs with no trace of a second orientation. The photograph reproduced here corresponds to the superposition of the diagrams of two fibres inclined at a small angle.

Figure 6. X-ray diagram of a single hemp fibre mercerized in 10% caustic soda. No trace of more than one orientation of cellulose chains is visible.

Figure 7 *a*, *b*. Photomicrographs of a transverse section of a stem of hemp in the fibre region, *a* in ordinary light and *b* in polarized light. The wall is isotropic, with the exception of the outer layer and, very occasionally, of the innermost layer.

Figure 8. Hemp fibres after treatment for 1 hr. with 10% caustic soda. Numerous transverse markings are clearly visible in the outer layers of the wall, and their appearance suggests folds rather than striations.

Figure 9. Secondary fibre treated with 55% sulphuric acid. "Spiral ballooning" is clearly visible in the upper part of the fibre. In the lower part the outer constricting band has become detached, and the fibre is here commencing to swell uniformly.

Plate 15

Figure 10. Fibre swollen in caustic soda and locally compressed. In the compressed region the wall has broken up into fibrils which are now inclined at a considerable angle to the fibre axis.

Figure 11. Hemp fibre showing "spiral ballooning" (upper third) which by locally pronounced swelling (centre) is becoming transformed into the more usual type (lower third).

Figure 12. More highly magnified view of a fibre in the condition illustrated in figure 9, plate 14.

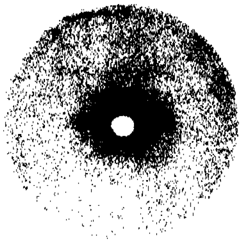

FIGURE 5

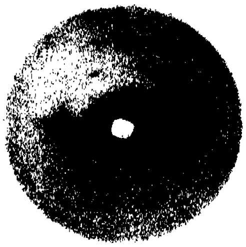

FIGURE 6

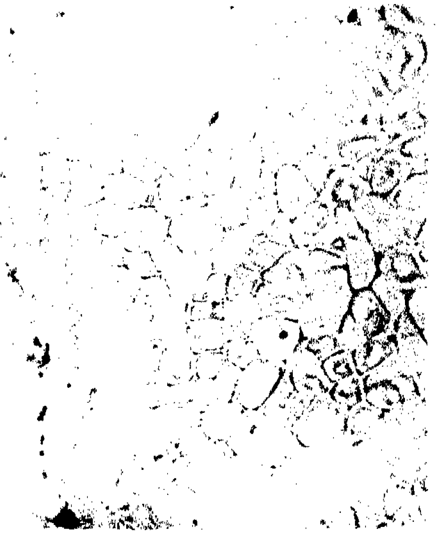

FIGURE 7*a*

FIGURE 7*b*

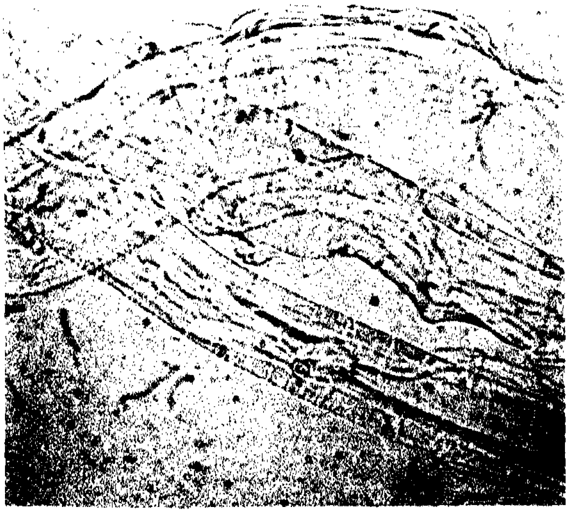

FIGURE 8

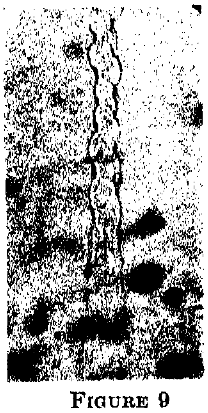

FIGURE 9

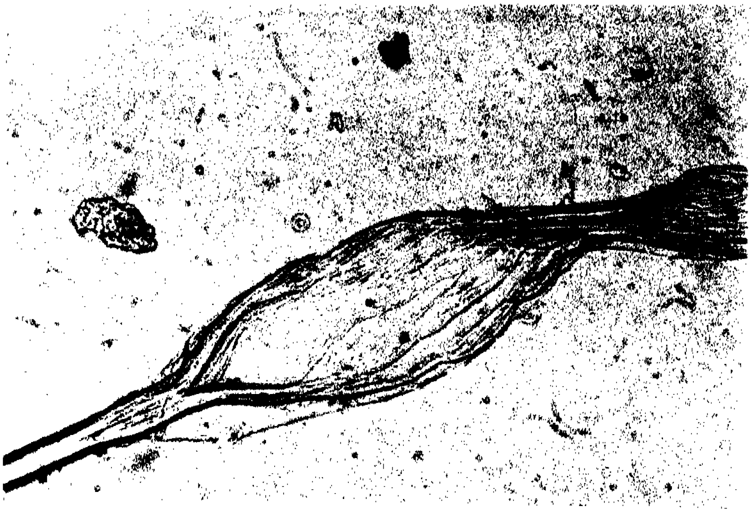

FIGURE 10

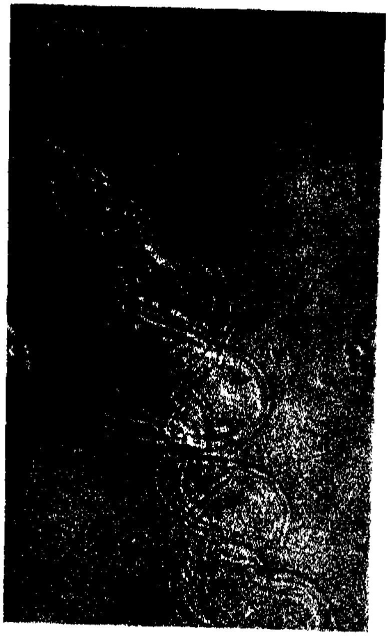

FIGURE 11

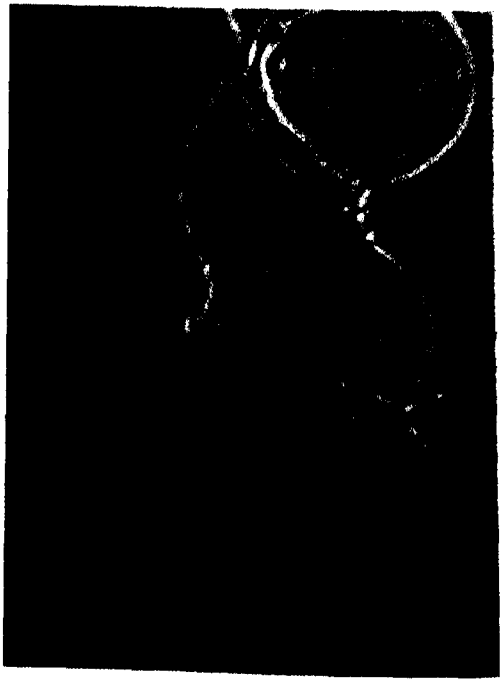

FIGURE 12

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The effect of adaptation on subjective brightness

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Introduction

Attempts have often been made to find a relation between stimulus and sensation magnitudes since the implications of the existence of absolute and differential thresholds, and theories of intensity discrimination, flicker sensitivity, resolving power, and so forth must depend largely on the view taken of this relation. Some of the problems have been discussed by Wright (1938).

Two principal methods have been employed in the case of vision: first, the direct estimation of sensation intensities as numerical multiples of one another (Richardson 1929; Maxwell 1929; Hopkinson 1939) or as intermediate between other sensation intensities (Gage 1934); and secondly, the integration of the $I/\Delta I$ curve, on the assumption that just noticeable differences at all points of the sensation-intensity range represent arithmetically equal sensation steps (Fechner 1858; Abribat 1935). Both these methods have been criticized on the general ground that sensation intensity is not a measurable quantity, and that the numerical results obtained are therefore illusory. In the case of the first type of experiment it is held that the numbers are merely names applied by convention to sensory intensities which can only be ranked in an ordinal series; and in the case of integration of ΔI's it is objected that there is no method of showing that j.n.d.'s at different sensation levels represent equal increments in sensation intensity. It has also been shown by Gage that even if the assumption of the equality of j.n.d.'s were correct the results obtained would be false, since the effect of adaptation in altering the sensation intensity after initial exposure to a changed stimulus is neglected.

These difficult issues are not raised by the present investigation, which employs a less bold method to indicate the relation of sensation to stimulus intensity in certain special cases. It is generally admitted that a judgement of equality can be made with regard to two sensation intensities, though it may be denied that one can be judged to be twice as great as the other; subjective photometry depends on such judgement of equality. This judgement of equal sensation intensities may be continually correlated with equality of stimuli where the state of adaptation of the sense organ or organs is the same throughout; but if the state of adaptation changes, or if two sense organs (e.g. the two eyes) are employed in different states of adaptation, the judgement of equality will not be correlated with physical equality. This method has been used by Beuttell (unpublished) and recently by Schouten and Ornstein (1939) to measure the (comparatively small) adaptive effect of glare sources. It permits simultaneous or immediately successive comparison and is therefore much more accurate than monocular memory matching.

The state of adaptation of the eye is determined by the light intensity to which it is exposed for a sufficient time, and though it re-adapts to any different illumination to which it is then exposed, its condition, for a second or so, is sufficiently determined by the previous adapting illumination to enable very interesting results to be obtained. To an approximation, then, the state of adaptation of the eye can be stated in terms of the illumination to which it has been adapted. The method of binocular comparison thus permits the establishment of all those combinations of states of adaptation and of test light intensities which give rise to equal sensation intensities. It also permits comparison between the conditions for constancy of absolute sensitivity and of differential sensitivity and acuity.

APPARATUS AND METHOD

1. *General requirements of apparatus*

One eye (the right in all these experiments) is to be exposed to any illumination until adaptation is complete, and the left eye at the same time brought into a fixed state of adaptation, constant through one series of readings, by exposure to darkness or to any illumination. The left eye is then to be exposed for 1 sec. to a variable illumination, adjusted in successive exposures until this illumination appears equal in brightness to the right-eye field. To enable this comparison to be made, the left-eye adapting field must be shut off 1 sec. previously, so that the brightness of the right-eye field alone, and not the fused brightness of the left and right adapting fields, may be taken as the standard for comparison with the left-eye test field; and similarly the right-eye field must be cut off while the left test field is exposed. (A totally dark field exposed to one eye is, of course, suppressed entirely, whereas two fields of nearly equal brightness fuse together to produce a brightness which may be intermediate between that of the two seen separately.) Schouten (1939) has recently confirmed that mutual adaptive effects between the two eyes are very small, and that pupil size is fixed for both eyes by the brightest light falling on either.

The presentation of these fields should be as rapid as possible, to avoid changes in the condition of adaptation of the left eye.

High physical brightnesses (e.g. 15,000 e.f.c.) are necessary, as many interesting adaptive effects occur at these levels. Other requirements militate against this; infra-red absorbing filters must be interposed to prevent ocular damage, and ground glass diffusing screens to produce an even field. Again, to facilitate brightness comparison the colour of the fields, on initial exposure, should approximate as closely as possible to the bluish white colour which light-sources having quite diverse spectral distributions attain after adaptation, owing to selective fatigue of the retina by preponderant wave-lengths. The field must also be large (e.g. 45°) to minimize spatial adaptation and "surround" effects (Lythgoe 1932; Lythgoe and Tansley 1929). The Maxwellian view is the most convenient method of fulfilling the greatest number of these requirements, and at the same time provides a fairly long optical path for the insertion of neutral filters, diaphragms, field stops, etc. An adjustment of inter-eyepiece distance is also required to suit different subjects, and must be without effect on the field illuminations. Scattered light must be reduced to a minimum, since it sets a limit to the range of illuminations obtainable with neutral filters (in the present case 7 log units with one light source).

2. *Details of apparatus*

A 100 W 200 V projector lamp at *a* (figure 1) illuminates a lens *b* of 18 mm. diameter and 16 mm. focal length. An eye placed at the conjugate focus of this lens sees it uniformly filled with light. Two Wratten neutral filters mounted in *B* glass can be inserted at *c*, field stops at *d*, and an infra-red absorbing filter of Calorex glass and a ground glass diffusing plate are fixed permanently at *e*. (The absorption of Calorex in the visible red also renders the spectral distribution of the light more like that of sunlight, and facilitates comparison between initial and adapted fields, as noted above.) The right eyepiece is supported by a bracket *f* rotating about the axis of the lamp *a*, to suit subjects with different inter-ocular distances. A mirror *g* reflects light from the projector lamp through a second similar eyepiece *h* with Calorex and ground glass at *k*, a filter frame at *l*, and a field stop at *m*. Another mirror *n* also reflects light through the iris diaphragm *o* and filter frame *p* into the left eyepiece; but this light is cut off, in the adapt position, by the mirror *g*, which is pivoted at *q* and held in the position shown in heavy lines by a spring *r*. A shutter *s* enables the left adapt field to be cut off and the right exposed alone; then the mirror *g* is rotated to the position shown by broken lines (*g*); it thus ceases to reflect light into the left eyepiece, and exposes the mirror *n*. The shutter *s* is actuated by a collar on the arm which rotates the mirror *g*, so that moving the hand down and across exposes both test fields in turn. An electric hair-drier at *t* ventilates the lamphouse *u* and prevents overheating of the filters. Internal screens are fitted to reduce scattered light. A 3 V 1 W bulb in an adapter constructed to bring its filament into the same position as that of the 100 W lamp, and provided with a cap of ground cellophane, could be substituted for the larger bulb for investigation of very low illuminations, where scattered light rendered the calibration incorrect. The optical parts were mounted on a plate of 5 mm. brass 13 cm. square, on a pedestal and tripod base. A chin rest was provided for the subject.

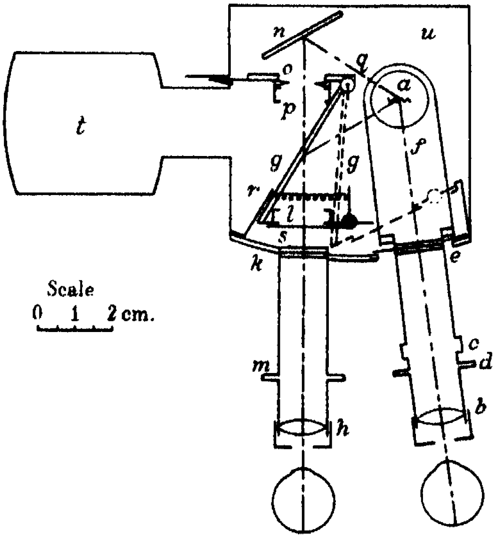

FIGURE 1. Diagram of apparatus. For description see text.

Calibration. The absolute and relative calibration of the different fields, and of the iris diaphragm scale was accomplished by a generator-type photocell clipped to each eyepiece in turn, used with a microammeter. The photocell and meter had previously been calibrated for the same "pupil" and field sizes by a diffusing plate illuminated by a 1000 c.p. projector lamp. The relative calibration of the fields was checked at intervals, and is accurate to ± 2 %. Absolute calibration of such fields is much more difficult, but less important, since there is probably no visual function to which an absolute intensity change of 50 % makes any significant difference. The calibration of the present instrument was checked by various methods, which agreed to within ± 20 %. The substitution of the 1 W bulb introduced a difference in wave-length composition of the light, and inevitable small differences in the distribution of light throughout the optical system; its use was more to indicate the trend of the lower parts of the curves than to give results comparable with those obtained with the 100 W lamp.

The maximum physical brightnesses obtainable were: right-eye field 15,000 e.f.c.; left-eye adapting field 7500 e.f.c.; left-eye test field 2500 e.f.c.

3. *Procedure*

The subject was seated with his head in the chin rest and asked to adjust the position of the rest and the distance of the eyepiece until both adapting fields were seen with maximum brightness and uniformity; the left-eye test field was then presented, and the subject told to readjust his head and the eyepiece until this also seemed uniform; on returning to the adapt position further adjustment could be made, until finally a position of uniformity for all fields had been found, with the head firmly fixed throughout. The subject was instructed not to attempt to make the adapting fields of the two eyes coincide, since this was quite unnecessary. Adaptation was then commenced. For left- and right-eye fields over 10 e.f.c. 2 min. adaptation was found sufficient to induce a stationary state; for adaptation to very low illuminations or to darkness, longer periods up to 20 min. were allowed. The experimenter then said "Ready—There—There", presenting first the right field alone, by lowering the shutter over the left adapt field, then the left test field alone by moving the mirror across. Each field was exposed for 1 sec.; the change-over occupied about $\frac{1}{8}$ sec. The subject then reported whether the second field appeared dimmer or brighter than the first. Periods of 5 sec. to 1 min. were allowed for recovery of adaptation between judgements; the longest periods were necessary when the left eye was dark adapted, and exposed to relatively high test illuminations. Insufficient recovery time showed itself as a steady drift in the readings. The point of equal brightness was found roughly by a "bracketing" method, as in firing range-finding shots; i.e. the iris diaphragm was set to points above and below the equality point, and converging upon it. Ascending and descending series were then used, as in the ordinary method of limits. The subject was instructed to say "doubtful" unless he was certain the second field was dimmer or brighter, since a large uncertainty or "hysteresis" region is much less perplexing in calculating the results than reversals (i.e. a series of judgements such as "dimmer, dimmer, brighter, dimmer, brighter, brighter" when the stimulus is being changed in one direction throughout).

The whole experiment consisted of six series, each showing the values of left-eye test illumination required to match adapted right-eye brightnesses ranging from $\frac{1}{1000}$ to 15,000 e.f.c. for a fixed left-eye adapting illumination. Each series occupied about 1 hr. The possibility of fatigue limited the experiment to sittings of this length, which permitted, on an average, only about 20 judgements at each right-eye illumination.

The complete experiment was performed on three subjects. Sample series were obtained from three others. One of the subjects in the main experiment is somewhat green-blind, and another slightly myopic; the others were normal. Cases of slight difference in brightness sensitivity between the two eyes were found.

All the subjects complained of colour differences, which made brightness comparison difficult, when the two eyes were in widely different states of adaptation. As already pointed out, this is due to the selective fatigue or colour adaptation of the retina, which tends to reduce various spectral distributions to a bluish white colour. It is possible by blue filters to render the initial colour of the field, exposed to a dark-adapted eye, very similar to this, but so much light is lost in the process as to make the experiment worthless. The colour difference in the present experiment was not a serious difficulty, and was reduced by the red absorption of the Calorex filters.

Another cause of difficulty was the different character of initial and adapted fields, quite apart from their brightness. An adapted field, be it of high or low intensity, acquires perfect uniformity and a watery, translucent quality. An initial field, exposed to a dark-adapted eye, has, on the contrary, a glowing, opaque appearance like white-hot iron, and curious inhomogeneities—bright patches and currents. Even this did not, however, seriously disturb the subjects in their judgement of equal or differing brightness.

Results

The results for one subject are shown in graphical form in figure 2. Each curve is taken with a fixed state of adaptation of the left eye, and shows (as ordinates) the illuminations presented for 1 sec. exposures to this eye, which appear equal in subjective brightness to the illuminations (as abscissae) to which the right eye is adapted. The ordinates thus represent the illuminations giving initial brightnesses, and the abscissae those giving adapted brightnesses, which are subjectively equal, for a fixed state of adaptation of the left eye. A family of such curves is shown for the different left-eye adapting illuminations marked against the curves.

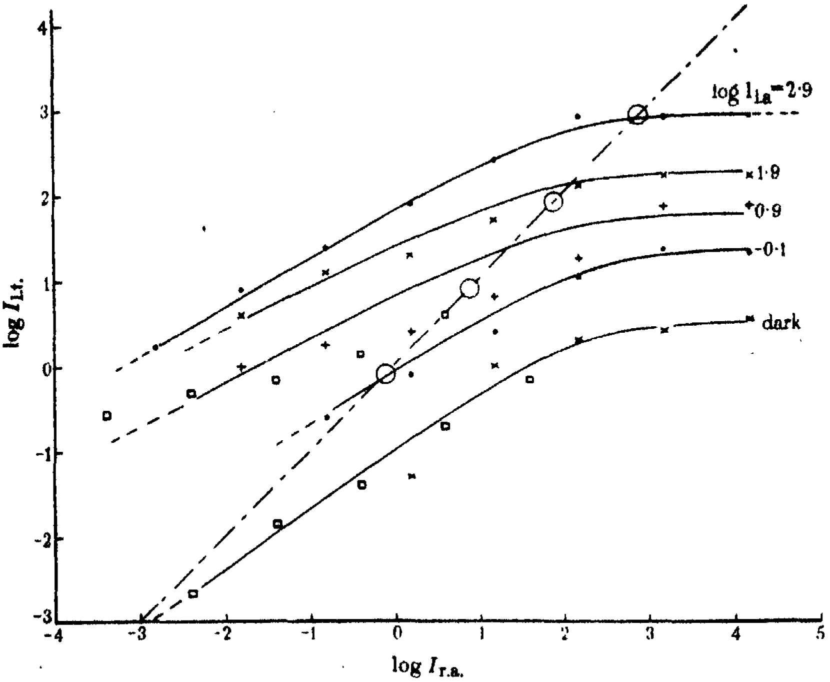

FIGURE 2. Relation between initial and adapted illuminations giving equal subjective brightness. Abscissae: right-eye adapting illuminations in log e.f.c. Ordinates: initial illuminations, presented to the left-eye when adapted to the illumination marked beside each curve, which appeared equal in brightness to the field to which the right eye was adapted. Circles show equal left- and right-eye adapting illuminations. The curves should ideally pass through them.

Four points may be noted in these curves.

First, they are very similar in general shape, but shifted steadily downwards with decreasing left-eye adapting illumination, as would be expected if the effect of adapting the left eye, within considerable limits, is simply to alter its range of sensitivity.

Secondly, all the curves rise very little above a right-eye adapted illumination of 100 e.f.c. and are apparently quite level above 1000–15,000 e.f.c., the upper limit of the apparatus. (A few readings were taken on one subject with a 250 W lamp and other alterations, giving a physical brightness of 75,000 e.f.c., and the curve was still found to be level. More detailed investigation was considered inadvisable owing to the intensity of the infra-red radiation passing into the eye.)

Thus no further increase in initial brightness to the left eye is required to match adapted brightnesses above 1000 e.f.c.; i.e. adapted subjective brightness increases little above 100 e.f.c. and is constant, within the limits of experimental error, above 1000 e.f.c. It is easy to show that this is not a false conclusion, due to inability to produce higher initial subjective brightness in the left eye for comparison, since a slight increase in left-eye test illumination is reported by the subject to render it very much brighter than the right-eye adapted brightness. Ample increase in left-eye subjective brightness is therefore available, but is not required to match the adapted right-eye brightness.

Thirdly, at illuminations below 100 e.f.c., adapted subjective brightness is smaller the smaller the illumination, but does not decline so rapidly as initial brightness. Thus, from $\frac{1}{1000}$ to 100 e.f.c. right-eye adapting brightness, the curve is approximately a straight line with a fixed slope of $x = 2y$, where x and y are the values of the abscissae and ordinates respectively in log units.

Fourthly, the magnitude of the effect of adaptation on subjective brightness may be seen from the fact that an initial illumination of 3 e.f.c. presented to the dark-adapted left eye is judged subjectively equal to the adapted brightness of 15,000 or 75,000 e.f.c. presented to the right eye.

The number of readings taken at each point was small, owing to the number of points requiring to be established during the course of an experiment, and the probable error of the values therefore somewhat large, but negligible in proportion to the magnitude of the effects obtained. Thus, the standard deviation varies from about 25–75 % of the mean value; to equate the subjective brightness of initial and adapted illuminations of 3 and 15,000 e.f.c. to within this error, means that the subjective brightness of illuminations differing 500,000 % is equated to within 75 % of one of those illuminations, or the standard deviation is only about $\frac{1}{6700}$ of the effect found, in this typical case. The scatter was slightly, but not markedly, greater when the eyes were in widely different states of adaptation, owing, apparently, to the differences between initial and adapted colour, the rapidly changing state of adaptation, when the left-eye test and adapting fields were very different, and to the dissimilar quality of the fields—the even brightness of an adapted field and the uneven, glowing character of an initial one.

The effect of field-size on subjective brightness is at present being investigated; some preliminary results indicate that reducing the right-eye field, at 1000 e.f.c., from 45 to 2° diameter, without altering its physical brightness, requires an increase in left-eye test illumination of the order of 30 % to balance it.

DISCUSSION

The two processes of bright and dark adaptation—the one producing a decrease in sensitivity during illumination, the other an increase in sensitivity in darkness—form a system which compensates subjective brightness for changes in illumination. This raises the problem to what degree, and over what range, this compensation is effective.

Absolute threshold measurements made under various adapting illuminations have been used by Nutting (1920) and Wright (1934; 1937) to indicate the sensitivity of the eye. They provide very valuable evidence, but introduce certain difficulties. First, the positive after-image (probably a type of after-discharge and therefore only indirectly connected with sensitivity) may mask the test patch. Secondly, only one curve relating absolute threshold to adapting illumination can be obtained, whereas the present method provides a family of curves at different left-eye adapting illuminations. Nutting's conclusions were that the absolute threshold is proportional to the adapting illumination from about 100 to 6000 e.f.c., and by extrapolation to 50,000 e.f.c., where he considers no further adaptation takes place. This suggests that sensitivity varies inversely as illumination over the range from 100 to 6000 e.f.c., and that compensation is therefore perfect and subjective brightness constant. This is in agreement with the present results, except that measurements at 75,000 e.f.c. do not show the upper limit to adaptation obtained by him by extrapolation. Nutting's results, and the present ones, are however incompatible with the classical "sensation curve" derived by simple integration of the area under the $I/\Delta I$ curve, completely ignoring adaptation. The existence of such adaptation renders it incorrect to assume that after any addition to the stimulus intensity the sensation remains at the higher level occasioned by this increase; consequently there is no doubt that the classical sensation curve is quite erroneous. (Integration of ΔI's at any given adapting illumination is probably more legitimate, but we still cannot be certain whether a j.n.d. corresponds to a certain absolute increase in sensation intensity or to a certain ratio of incremental to main sensation intensity.)

Wright (1934) predicted from some binocular comparison experiments that subjective brightness should be nearly constant over the range for which $\Delta I/I$ is constant, and suggested a theory of brightness discrimination depending upon this. He argued that where the sensation intensity due to a range of main illuminations is rendered constant, owing to adaptation, the sensation increment due to a certain fractional increase in these illuminations must also be constant. He later, however (1935), abandoned this theory of intensity discrimination on finding that $\Delta I/I$ might be practically constant over a tenfold change in test illumination, for a fixed state of adaptation. Clearly, the subjective brightness resulting from the test illuminations varies greatly over this range.

We now see that he was correct in supposing that adapted subjective brightness is constant* over the higher intensity range, though not over so large a range as that over which $\Delta I/I$ is approximately constant. Further investigation of brightness discrimination at illuminations above and below those to which the eye is adapted (Craik 1938) has confirmed that $\Delta I/I$ is indeed almost constant where subjective brightness is constant, but that $\Delta I/I$ may still be almost constant where subjective brightness varies. This behaviour of the mechanism of intensity discrimination is hard to explain on a purely photochemical theory, whereas the behaviour of subjective brightness fits well with such a theory.

According to the photochemical theory of Hecht (1923), the sensory response is determined by the product of light intensity and concentration of photosensitive substance, the latter in turn being determined by the equilibrium velocities of the supply and dissociation of this sensitive substance, and therefore by the light intensity, when this is constant for some time. Since rate of supply = rate of dissociation† = sensory response at equilibrium, we can infer that each of these is constant when adapted sensory response is constant, i.e. above 100 or 1000 e.f.c.; below this the rate of supply decreases, though we cannot say how fast. A twofold decrease in initial intensity = a tenfold decrease in adapted intensity, suggesting that there is fivefold or partial compensation here. Onefold or ineffective compensation presumably takes place at the lowest illuminations, when the limit of dark adaptation has been reached. Hecht (1936) asserted that the state of adaptation between 30 and 1500 e.f.c. is constant, but his brilliant revised theory of intensity discrimination (1935) leaves room for this decrease of response in compensation for increased illumination, though he does not predict the extent of this decrease and the consequent final level of the adapted brightness.

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* A 2° field, as already noted, may appear subjectively brighter than a 45° field at the same illumination; if it requires a 30 % increase in left-eye initial illumination to match it, we may say very roughly that the dark surround has caused the right eye, at equilibrium, to assume a state of adaptation appropriate to an illumination 30 % lower than that of the 2° field to which it is actually exposed. Further investigation is however required to show whether the effect may not be due in part to changes in pupil size.

† Positive after-images suggest that momentary concentration of photoproduct, rather than rate of dissociation, determines the response, but for the present purpose the difference is negligible.

Hartline's evidence (1938) that the adapted response is transmitted by only one type of fibre further suggests that it may be a fairly close copy of the photochemical events, since at least the effect is not confused by the response of several types of fibre having, perhaps, different excitabilities. The changes in subjective brightness investigated in the present experiments are relatively slow, again suggesting their photochemical origin. There is recent evidence of very rapid adaptive processes, occupying only $\frac{1}{25}$ sec. (Schouten and Ornstein 1939). They are, however, small compared with the present effect; indeed the magnitude of these changes—many thousandfold—is very difficult to account for on any but photochemical lines.

Difficulties arise, however, in trying to explain brightness discrimination in the same way. Hecht's theory (1935) seems at first to meet the case. It suggests a way in which keen discrimination may be possible on the basis of initial changes in brightness, and accounts for constancy of $\Delta I/I$ over the range of illuminations above 1 e.f.c.* But it does not explain the very small variation in $\Delta I/I$ at test illuminations somewhat greater or smaller than that to which the eye is adapted. A certain range of assumptions as to the photochemical counterpart of the j.n.d. and of the relation between light intensity and dissociation rate for a constant concentration of sensitive substance is possible, but we should still expect the *same* relation to hold throughout; constancy of $\Delta I/I$ over a considerable range of test illuminations, with deterioration both above and below this range, is hard to account for. Thus, if a j.n.d. represents a constant absolute increment in dissociation rate we should expect differential sensitivity to be improved at test illuminations above the adapting one, which is not the case except for very low adapting illuminations, where an alternative explanation is available (see end of discussion). On the other hand, it would account for the decrease in differential sensitivity at illuminations below the adapting one. The simplest alternative assumption, that the j.n.d. represents a constant ratio of new to previous dissociation rate, would account for the constancy of $\Delta I/I$ at test illuminations somewhat above or below the adapting illumination, but predicts that this should hold for an indefinite distance above the adapting illumination, and below it, until the absolute threshold is reached. On the contrary, differential sensitivity deteriorates steadily at illuminations considerably above or below the adapting one. Even if we postulate some more complicated relation between the j.n.d. and its photochemical basis, it is extremely hard to account, on purely photochemical lines, for deterioration in differential sensitivity *both* above and below the adapting illumination.

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* Hecht (1935, p. 770) gives an equation "which says that the initial rate of photochemical decomposition on introduction of the higher intensity to the photochemical system at the stationary state is proportional to ΔI times the concentration of sensitive material at the stationary state", and again "Let us assume that for the intensity $I+\Delta I$ to be distinguished from the intensity I, the initial rate of decomposition of the sensitive material on the addition of ΔI is the same, no matter what the intensity I may have been." Then if, for a j.n.d., $\Delta I/I$ is constant over a certain range of test-and-adapt illuminations, or $\Delta I \propto I$, the concentration of sensitive material, at equilibrium, must vary as some inverse function of the illumination over the whole of this range. This is unlikely, since from 1–100 e.f.c. (where $\Delta I/I$ is constant) the compensation by photochemical adaptation is imperfect, as measured by subjective brightness, whereas above 1000 e.f.c. (where $\Delta I/I$ is still almost constant) it is perfect.

As pointed out previously (Craik 1938) we must not regard brightness discrimination as being proportional to the actual value of the adapted response; adaptation rather sets the eye to a certain *range* of sensitivity, as one might set a voltmeter to a 10 or 100 V range (cf. Lythgoe 1936). The adapting illumination is not equivalent to the single reference point of a null-reading instrument; it is the indication of the range of sensitivity to which the eye has been set. This "range-setting" bears many signs of being a photochemical process, as remarked above, but the response within the range seems rather different in nature; its features are a central region of "linearity" and inferior response at the ends, with signs of an "overload" effect at the upper end. (The failure of acuity at test illuminations far above the adapting one (Craik 1939) gives further subjective evidence of such an effect.) It recalls the behaviour of a voltmeter which has a good response over the middle of its scale, while the "stickiness" of its needle masks minute ratios of change at the lower end, and the jamming of the needle against the upper end-stop prevents further increases of voltage being measurable when it is overloaded. The overload effect may be neural or electrical; the "silent period" (Hartline and Graham 1932) and dropping of the initial-frequency curve at high illuminations (Hartline 1938) affords evidence of some type of refractory phase; while Granit (1937) has found in the retinal potential an "amplification of potential differences" under optimal adaptation conditions which closely parallels the subjective data on testing at illuminations equal to or different from those to which the eye is adapted. Again, the effect may possibly be connected with distribution of thresholds among individual receptors, as in Hecht's theory of acuity (1928). These conceptions may, indeed, be mutually compatible as expressions of the superposition of neural upon photochemical effects. We may suppose that the stimulus to the neural mechanism is a certain concentration of photoproduct, whether achieved by a large concentration of photosensitive substance and a small illumination, or by a small concentration of sensitive substance and a high illumination.* Hence, we may suppose, arise the large, slow changes in sensitivity. To this stimulus of photoproduct concentration some receptors or nerve-endings are perhaps more sensitive than others, some more rapidly adapting than others, and some (the off-fibres), perhaps owing to their spatial arrangement, polarized in the sense required for excitation only by a decrease and not by an increase in photoproduct concentration. The total response of all these fibres to increases and decreases in photoproduct concentration and therefore in illumination appears to be, over a certain range, in accord with the Weber-Fechner law, and closely imitates the intensity discrimination of the eye adapted to each test illumination in turn, where the obedience to the Weber-Fechner law is probably due in the main, at high illuminations, to the reduction of the mean sensation response to a constant level by photochemical adaptation. The two types of response, the initial, in which several types of fibre are firing, and the adapted, in which only one type is involved, seem further to imitate one another, for brightness matches between the two resulting sensations are possible, despite a certain turbulence in the former.

In conclusion, probably the simplest way to visualize the changes taking place in subjective brightness as a result of adaptation and alteration in the illumination, is to consider the effect of exposing the dark-adapted eye to an illumination below the absolute threshold (say 1/1,000,000 e.f.c.) and increasing it suddenly once every minute to ten times its value at that moment. The physical brightness will thus rise, staircase fashion, from 1/1,000,000 to 100,000 e.f.c. in the course of 11 min. (curve *I*, figure 3). Sensation intensity (curve *S*) will not rise in the same way. It can be indicated diagrammatically by a non-numerical ordinate scale. At first, the subjective brightness will be zero, until the absolute threshold is reached; then it will begin to rise at every step, but each rise will be small (for differential brightness sensitivity is poor in this region); after each increase in illumination the subjective brightness will sink back slightly, owing to bright adaptation, but not to the same level as before; for the present experiment shows that subjective brightness rises over this range. At 1 e.f.c. the sensation steps resulting from each stimulus increase have become much larger (as is indicated by the greater differential brightness sensitivity), but after each increase the brightness falls to a lower value, still somewhat higher than its value before the last stimulus increase. Above 100 e.f.c. the size of the initial sensation steps resulting from each stimulus increase is probably the same as at 1 e.f.c., but the decline of sensation intensity on continued exposure is much more marked; above 1000 e.f.c. it is so great as to compensate exactly for each increase in illumination, so that the adapted response is constant above this level, as indicated by the broken line.

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* This equivalence of light intensity and of photochemical sensitivity, so long as their product is constant, may not be rigidly true of latency (Lythgoe 1938).

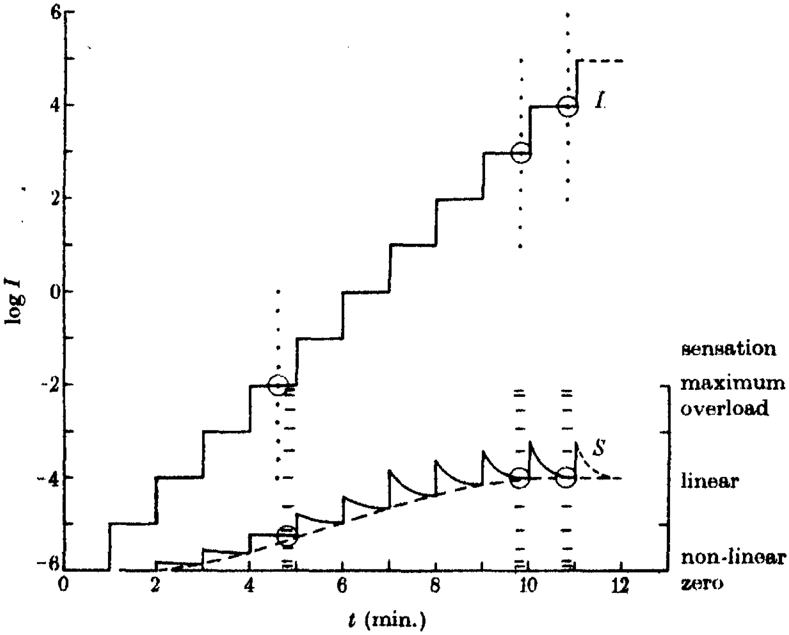

FIGURE 3. Diagrammatic representation of the relation between stimulus and sensation intensity. Abscissae: time. At the end of each minute the stimulus intensity is increased by a factor of 10, as shown by curve I. Ordinates: on the left side, stimulus in e.f.c.; on the right side, non-numerical scale of sensation intensity or subjective brightness. Curve S shows the relation of this to stimulus intensity, including the "saturation" effect, and the response (short dashes) to logarithmically equal changes in stimulus intensity (dots on curve I). This indicates the regions of "linear" sensation response, of "alinearity" above and below this, and of "overload" at the maximum.

We can also suggest the place in the scheme of brightness discrimination at illuminations above or below that to which the eye is adapted. If the eye is adapted, say, to 1000 or 10,000 e.f.c., brief exposures to test illuminations spaced equally on a logarithmic scale above and below this will show that differential sensitivity is still fairly good for one log unit above or below the adapting illumination but deteriorates sharply above or below this. On the assumption that a j.n.d. represents a constant sensation-step, and with these precautions concerning adaptation, we can integrate ΔI's and obtain the sensation intensities as shown by the spacing of the dashes above and below the sensation curve. These suggest the non-linear increase of sensation intensity with very high initial illuminations, which we have called the overload effect. It has also been found (Craik 1938) that at very low adapting illuminations the maximum differential sensitivity occurred not at this but at a somewhat higher test illumination. In figure 3 the decline of adapted subjective brightness at $\frac{1}{100}$ e.f.c. shows that compensation is imperfect at this point; i.e. the "adapting point" has shifted down on the subjective brightness range, instead of keeping a constant position. The consequence is that there will be a greater range of "linear response" above than below the adapting point, and brightness discrimination will be somewhat better at a higher test illumination, as suggested by the wide spacing of the dashes. (The actual situation seems to be somewhat more complicated owing to the combination of rod and cone adaptation in normal vision. Peripheral vision shows a much greater "overload effect" at high illuminations, which suggests that this is mainly a rod effect, as was proposed in the paper quoted.)

Incidentally, the fact that subjective brightness is compensated for changes in general illumination, perfectly over the range from 1000 to 15,000 e.f.c. and to a considerable extent down to 1 e.f.c. or lower, suggests that this type of adaptation may play a considerable part in the phenomena of brightness constancy, i.e. the tendency for the relative brightnesses of objects to remain fairly constant under widely different general illuminations. This role for adaptation was originally proposed by Hering (1905), but has received little sympathy in more recent psychological treatments of constancy (Katz 1935; Koffka 1935).

SUMMARY

Subjective brightness has been investigated by binocular matching. The adapted response "saturates" at 1000 e.f.c.; below this it decreases less rapidly than initial brightness in the proportion, roughly, of 1:2 on a log scale of stimulus intensities. Adaptation thus compensates subjective brightness for slow changes in illumination, perfectly above 1000 e.f.c. and less perfectly below this. The adapted level of subjective brightness is relatively low; e.g. the initial brightness of 3 e.f.c. exposed to the dark-

adapted eye equals that of 15,000 e.f.c. to an eye adapted to that illumination.

The data on subjective brightness fit well into a photochemical theory of vision. The difficulties of accounting similarly for some of the features of brightness discrimination under a fixed adapting illumination are discussed, and it is suggested that effects due to the neural system are superimposed on those due to the photochemical mechanism, and that this neural system involves nerve-endings of different types whose behaviour under a range of illuminations, denoted as the lower non-linear, linear, and upper non-linear or overload ranges, is better described in terms of their own excitability, adaptive and refractory period characteristics than in terms of the underlying photochemical mechanism.

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The photodynamic activity of the tissues of mice treated with 3:4-benzpyrene

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The presence of 3:4-benzpyrene can be detected in dilutions of the order of 1 in 100 million, by means of the photodynamic action on *Paramoecium* (Mottram and Doniach 1939; Doniach 1939). Moreover, this photodynamic action is little altered by the presence of other substances; the method therefore is very suitable for its detection in the tissues of animals which have been either painted or inoculated with benzpyrene.

The procedure adopted was to take about 50 mg. of tissue, mince it, spread it on a glass slide, dry it over phosphorus pentoxide at reduced pressure, weigh the dry powder and add redistilled acetone, ½ c.c./10 mg. of dried tissue: this was then boiled for 2 min. by immersion in hot water; the acetone was then poured off and water added to the acetone extract, 0·5 c.c./10 mg. of tissue taken; the acetone was next boiled off at reduced pressure, and any loss of water made up.

Thus was obtained a watery emulsion of the lipoids of the tissue with any benzpyrene which might be present. The final dilution was equivalent to 1 in 50 of the dried tissue.

The photodynamic activity of the emulsion was tested by adding an equal volume of a culture of *Paramoecium*, thus making the dilution 1 in 100, placing a few drops in a well slide and exposing to a mercury vapour lamp, under standard conditions as previously described (Mottram and Doniach 1939). Control cultures were kept in the dark for 2 hr. so as to exclude any toxic action on the *Paramoecium*. In only two cases was a toxic action found, and these were excluded.

The photodynamic activity is measured by the time taken to kill the *Paramoecium* under the light. If the *Paramoecium* are not dead after 60 min. exposure, the extract of the tissues was considered to be without photodynamic activity.

The tissues of ten normal white mice (Bagg strain) and of ten mixed coloured mice (mixed strain) were examined, all with negative results.

In the case of mice painted or inoculated with benzpyrene, negative results were obtained with the following tissues: heart, muscle, thymus, brain, spleen, kidney, submaxillary gland, skin (other than the painted skin), fat (other than fat close to the inoculated benzpyrene), testes, uterus; the pancreas and the intestines gave rise to toxic emulsions.

In contrast to this, positive findings were obtained in the case of four tissues: the painted skin, the inoculated subcutaneous tissue, liver and lungs.

In the case of the painted skin, positive results were obtained after repeated washing and scrubbing of the surface with benzene in an attempt to remove any benzpyrene which lay on the surface; also positive results were obtained for as long as a month after painting had stopped; longer periods were not tried.

In the case of the liver and lung, the results obtained are given in detail in tables 1 and 2, dealing with painted mice, and with inoculated mice.

The painted mice were painted on the mid-scapular region over a length of skin of about ½ in., twice a week with a 0·5 % solution of 3:4-benzpyrene in benzene. About 0·015 c.c. was the amount applied at each painting; the inoculated mice received 1 mg. of benzpyrene in 0·5 c.c. of olive oil into each flank; the mice used were of two strains, a white Bagg strain and a strain of mixed coloured mice. Referring to the table, it is seen that in about one-third of the white mice (eleven out of thirty-six) and in about one-fifth of the coloured mice (seven out of thirty-six) the presence of photodynamic material was found in the lungs. The livers were found to be active in about one-third of the white mice (thirteen out of thirty-eight) and in about half the coloured mice (twenty-one out of thirty-seven). The figures are too small to make any further analysis.

DISCUSSION

Many investigators have observed the production of pulmonary tumours in mice which had been painted or inoculated with blastogenic hydrocarbons. These lung tumours especially occur in strains of mice which have a tendency to their spontaneous occurrence. Andervont has shown in a series of papers (1937, 1938, 1939) that the susceptibility of the lung to tumour formation following the injection of 1:2:5:6-dibenzanthracene is inherited in a dominant manner. Andervont (1938) has also described the production of liver tumours in mice injected intravenously or subcutaneously with serum dispersions of 1:2:5:6-dibenzanthracene and with lard solution injected subcutaneously. In these experiments both lung and liver tumours occurred in mice which had no tendency to exhibit spontaneous lung tumours. In his last paper on induced pulmonary tumours (1939), the dominant nature of the inheritance does not hold, since hybrids were more susceptible than either of the parent strains.

TABLE 1

White mice				Coloured mice			
Days painted	Days after last painting	Lung	Liver	Days painted	Days after last painting	Lung	Liver
9	2	Nil	Nil	20	2	Nil	11
13	2	—	Nil	21	3	Nil	31
13	2	—	Nil	28	2	Nil	24
14	1	Nil	Nil	28	2	Nil	Nil
14	1	Nil	Nil	96	0	Nil	Nil
16	1	Nil	15	96	2	39	35
76	0	Nil	55	96	1	Nil	6
76	1	Nil	Nil	96	3	8	Nil
76	2	Nil	Nil	96	1	Nil	3
76	3	Nil	Nil	96	4	Nil	8
98	1	24	Nil	96	1	Nil	Nil
98	2	Nil	Nil	96	2	12	Nil
98	3	Nil	Nil	96	3	Nil	17
125	4	16	Nil	96	0	Nil	41
130	1	Nil	Nil	96	1	Nil	Nil
134	4	Nil	6	96	2	Nil	Nil
136	2	22	23	96	4	Nil	Nil
139	3	28	Nil				
141	1	Nil	Nil				
143	3	Nil	Nil				
146	3	39	Nil				
147	1	49	Nil				
149	2	8	33				
150	0	16	52				

Table 1 deals with painted mice and table 2 with inoculated mice. The first column in table 1 gives the number of days the animal had been painted at the time when it was killed and its tissues taken. The second column gives the number of days after the last painting that the animal was killed. Columns under lung and liver give the time in minutes required to kill the *Paramoecium* exposed to the light. The columns in table 2 are like the first, third and fourth of table 1. The expression "Nil" means that the specimen was exposed to the light for an hour without killing the *Paramoecium*.

It is evident that both lung and liver tumours are induced in mice treated with blastogenic hydrocarbons applied at distant sites. I am not aware that such treatment results in tumours in other tissues distant from the site of application. One might therefore expect to find evidence of the presence of these hydrocarbons only in the lungs and liver: at any rate, this was the case, since only in these two tissues was a photodynamic substance detected. If this had been found with painted mice alone, the possibility of the animal having either breathed or swallowed the hydrocarbon could not be excluded, but it occurred also with inoculated mice. From this, one must conclude that the 3:4-benzpyrene does not remain localized at the site of inoculation (it appears in the urine in a modified form (Mottram 1939)), that it is able to pass to the liver and lung and to become localized there in higher concentration than in most other tissues, and there to determine the occurrence of tumours.

TABLE 2

White mice			Coloured mice		
Days after inoculation	Lung	Liver	Days after inoculation	Lung	Liver
39	39	26	3	Nil	50
54	Nil	23	4	Nil	22
54	15	24	6	Nil	25
68	26	39	7	37	37
68	Nil	53	7	Nil	19
74	Nil	18	14	Nil	55
74	Nil	Nil	75	Nil	Nil
74	Nil	Nil	75	58	Nil
74	Nil	Nil	75	Nil	Nil
74	Nil	Nil	75	Nil	Nil
74	Nil	Nil	75	Nil	Nil
82	Nil	13	75	—	Nil
82	Nil	Nil	75	Nil	Nil
82	Nil	Nil	75	41	34
			75	Nil	23
			75	Nil	Nil
			75	Nil	53
			75	Nil	28
			75	55	22
			75	Nil	10

See note under table 1.

Perhaps susceptibility to lung tumours means a greater tendency to localization; it is to be noted that in the present series of mice positive results in the case of the lungs occurred with the white mice in one-third of the cases and in the coloured mice in one-fifth; also it is known that the white mice (Bagg strain) are more prone to lung tumours than ordinary mixed coloured mice.

CONCLUSIONS

1. The tissues of mice painted and inoculated with 3:4-benzpyrene were examined for the presence of photodynamic substances.

2. The presence of photodynamic substance was used as a measure of the presence of benzpyrene.

3. All the tissues of normal mice and most of the tissues of the treated mice showed no photodynamic activity.

4. Of the treated mice, the liver and lung alone showed photodynamic activity, apart from the tissues at the site of application of the hydrocarbon.

5. The high occurrence of lung and liver tumours in treated mice seems therefore to be due to a local concentration of the blastogenic hydrocarbons in these two tissues.

6. Susceptibility to lung tumours in treated mice might therefore be due to a special tendency for the hydrocarbons to accumulate in the lungs.

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SUMMARY

The photodynamic action of 3:4-benzpyrene on *Paramoecium* occurs in dilutions of the order of one part in a hundred million. By this means its presence has been looked for in the tissues of mice either painted or inoculated with this hydrocarbon. Apart from the site of its application it was found to be present only in the liver and the lungs. It is noteworthy that these are the only two sites apart from its place of application where tumours have been observed to occur in mice treated with carcinogenic agents. It appears therefore that the reason why tumours occur in the liver and the lungs is because the hydrocarbon accumulates in these two tissues.

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The nature of the oestrogenic substances produced during the demethylation of anethole

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The discovery of oestrogenic activity in synthetic substances not possessing the phenanthrene nucleus led eventually to interest in derivatives of stilbene, notably 4 : 4′-dihydroxy-stilbene, and in para alkyl substituted phenols (Dodds and Lawson 1937*a*; Dodds, Fitzgerald and Lawson 1937). It was noted that *p*-propenyl phenol (anol) was a member of the latter series but in addition possessed structural similarity to the former. The substance was accordingly prepared and tested for oestrogenic activity, the potency being found of the same order as that of oestrone (Dodds and Lawson 1937*b*).

Following this publication numerous workers repeated the preparation, but reports on the activity were conflicting. Confirmation of the original announcement was forthcoming from several laboratories but others were quite unable to obtain similar results. A reinvestigation of the subject showed that while certain batches of crystalline material had high oestrogenic activity, others were very much less active.

Later experiments demonstrated that the mother-liquor from chloroform crystallization of crude anol contained a highly active substance, and the interesting observation was made that anol which was completely inactive in doses of 10 mg. in rats yielded an active gummy product on heating with potassium hydroxide and alcohol for 15 hr. at 180–200° C. The opinion was advanced that the high activity of some specimens of anol was due to co-separation of a highly active by-product, the by-product being possibly an anol polymer (Dodds and Lawson 1937*c*).

Investigation into anol polymers resulted in the preparation of a di-anol, 1 : 4-di-(*p*-hydroxyphenyl)-2-methyl-1-pentene, by the demethylation of "isoanethole" (Goodall and Haworth 1930). This di-anol produced oestrus in castrated female rats in doses of 0·1 mg. (Campbell, Dodds and Lawson 1938*a*). An attempt was then made to determine whether this phenol occurred among the anol by-products. This attempt proved successful.

Oxidation of an oily fraction of the methylated by-products yielded both anisic acid and 1-(*p*-methoxyphenyl) *n*-propyl methyl ketone. Conditions for this oxidation were based on those of Goodall and Haworth (1930), who had used the method to prove the structure of "isoanethole". It was also found that a lower boiling fraction of the by-products contained *p*-*n*-propyl phenol, which was identified by means of its 3 : 5-dinitrobenzoyl derivative. This evidence of hydrogenation was later found of considerable importance, and in this respect it was noted that the gases produced during the action of potassium hydroxide and alcohol on anethole in an autoclave contained a high proportion of hydrogen.

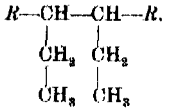
After a short time, Dodds, Golberg, Lawson and Robinson (1938) published an announcement of a series of compounds including 4 : 4′-dihydroxy-α : β-diethyl stilbene, which substance was found to be active in doses of between 0·0003 and 0·0004 mg. They pointed out that this substance was a possible anol dimeride.

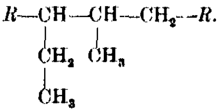
The oxidation experiment which resulted in the identification of 1 : 4-di-(*p*-methoxyphenyl)-2-methyl-1-pentene in the methylated anol by-products also indicated that the same fraction contained a part resistant to oxidation. After repeated oxidation this remained unchanged and examination showed it to be saturated and to have the molecular formula of a dihydro-dianethole. Its oestrogenic activity was also found to be double that of the original, partly unsaturated, fraction from which it was obtained. On standing, this saturated oil deposited crystals which also possessed the character and molecular formula of a dihydro-dianethole, and on demethylation, yielded a crystalline phenol, a dihydro-dianol, which proved to be fully active in doses of 0·0002 mg. in rats. This dihydro-dianol melted at 184°.

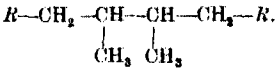
The oily mother-liquor, from which these crystals had separated, was demethylated and a gum obtained which partly crystallized. The crystals were collected and purified. They melted at 122° and were active in doses of 1 mg. This product was also a dihydro-dianol.

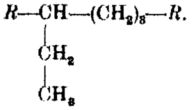
Two crystalline dihydro-dianols thus required identification, and the difficulty of determining the structures of compounds of this type by analytical methods was realized. It was decided to attempt, if necessary, synthesis of the six probable isomers which were expected to include the two in question.

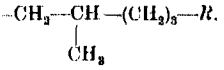
It was considered that the dihydro-dianols resulted from condensation of two molecules of anol or anethole, although the isomeric *p*-allyl phenol (chavicol) or its methyl ether (esdragol) might play some part. **Possible** products from such condensations taking place under hydrogenating conditions would be:

A.

$R-\underset{\substack{|\\ CH_2\\ |\\ CH_3}}{CH}-\underset{\substack{|\\ CH_2\\ |\\ CH_3}}{CH}-R$

B.

$R-\underset{\substack{|\\ CH_2\\ |\\ CH_3}}{CH}-\underset{\substack{|\\ CH_3}}{CH}-CH_2-R$

C.

$R-CH_2-\underset{\substack{|\\ CH_3}}{CH}-\underset{\substack{|\\ CH_3}}{CH}-CH_2-R$

D.

$R-\underset{\substack{|\\ CH_2\\ |\\ CH_3}}{CH}-(CH_2)_3-R$

E.

$R-CH_2-\underset{\substack{|\\ CH_3}}{CH}-(CH_2)_3-R$

F. $R-(CH_2)_6-R$

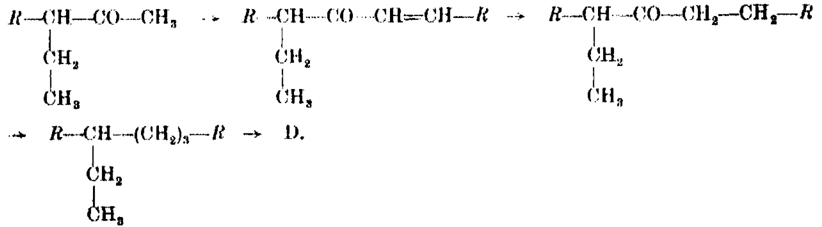
$R = p$-hydroxyphenyl.

Isomer F could only result from condensation of two molecules of chavicol, a most unlikely occurrence since the presence of chavicol in the smallest quantity was entirely conjectural and would be dependent on the usual isomerisation of allyl to propenyl being a balanced reaction and hence capable of proceeding in the reverse direction. The methyl ether of this isomer had been obtained as the product of hydrogenation of an esdragol dimeride by J. M. v. d. Zanden (1938), who also synthesized it. This dihydro-di-esdragol was synthesized by a new method and the product of its demethylation found to be a crystalline solid melting at 142° and showing no oestrogenic activity in doses of 100 mg.

Isomer A possessed an accumulation of theoretical evidence in its favour. The high activity of 4 : 4-dihydroxy-α : β-diethyl stilbene was outstanding in this respect. It is interesting to note that Serini and Steinruck (1937) mentioned the isolation of substances of this type from the products of demethylation of anethole with ethyl or n-propyl iodide. Positive evidence for the diphenylethane type of structure was supplied by Dr R. K. Callow, who showed that the very active dihydro-dianol (m.p. 184°) possessed an absorption spectrum different from that of 4 : 4′-dihydroxy-α : β-diethyl stilbene, but closely resembling that of 4 : 4′-dihydroxy-α : β-diphenyl ethane. Doubtless the ethylenic double bond makes comparison of a stilbene derivative with a saturated compound of little use. Dodds, Golberg, Lawson and Robinson (1938) had included 4 : 4′-dihydroxy-γ : δ-diphenyl-n-hexane among the compounds announced in the preliminary publication which included the corresponding stilbene derivative, but described the hexane as an oil. Since on stereochemical grounds two isomers, meso and racemic, would be expected, hydrogenation of 4 : 4′-dihydroxy-α : β-diethyl stilbene was carried out, yielding with difficulty, a product melting at 128°, active in doses of 0·1 mg. and not identical with either of the two dihydro-dianols. This structure was, for a time, abandoned, and attention directed toward the remaining four structures.

Isomer B was readily obtained by hydrogenation of "isoanethole" and demethylation of the oily methyl ether. The phenol melted at 128° and proved to be identical with the lower melting member of the two dihydro-dianols under consideration (m.p. 122°).

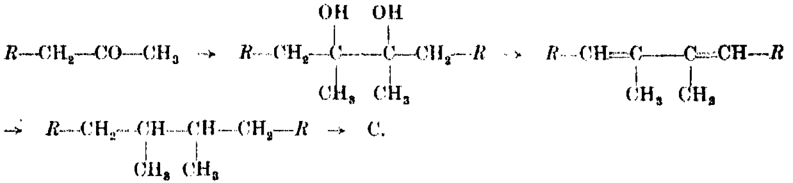
Isomer D was synthesized according to the scheme depicted below, and was obtained as a crystalline solid melting at 98° and active in doses of 5 mg.:



$$R{-}\underset{\substack{|\\ CH_2\\ |\\ CH_3}}{CH}{-}CO{-}CH_3 \rightarrow R{-}\underset{\substack{|\\ CH_2\\ |\\ CH_3}}{CH}{-}CO{-}CH{=}CH{-}R \rightarrow R{-}\underset{\substack{|\\ CH_2\\ |\\ CH_3}}{CH}{-}CO{-}CH_2{-}CH_2{-}R \rightarrow R{-}\underset{\substack{|\\ CH_2\\ |\\ CH_3}}{CH}{-}(CH_2)_3{-}R \rightarrow D.$$

$R = p$-methoxyphenyl.

Isomer C was synthesized as below and proved to be a solid melting at 152° and active in doses of 10 mg.



$$R{-}CH_2{-}CO{-}CH_3 \rightarrow R{-}CH_2{-}\underset{\substack{|\\ CH_3}}{\overset{\substack{OH\\ |}}{C}}{-}\underset{\substack{|\\ CH_3}}{\overset{\substack{OH\\ |}}{C}}{-}CH_2{-}R \rightarrow R{-}CH{=}\underset{\substack{|\\ CH_3}}{C}{-}\underset{\substack{|\\ CH_3}}{C}{=}CH{-}R \rightarrow R{-}CH_2{-}\underset{\substack{|\\ CH_3}}{CH}{-}\underset{\substack{|\\ CH_3}}{CH}{-}CH_2{-}R \rightarrow C.$$

$R = p$-methoxyphenyl.

At this point in the research a re-investigation of structure A was planned. Hydrogenation of a new specimen of 4 : 4′-dihydroxyl-α : β-diethyl stilbene gave a product, again with difficulty, which by fractional crystallization yielded two crystalline substances, one of which was identical with the highly active dihydro-dianol (m.p. 184°). This was in full accordance with theoretical anticipation. Hydrogenation of 4 : 4′-dihydroxy-γ : δ-diphenyl hexadiene (Dodds, Golberg, Lawson and Robinson 1938) gave an almost quantitative yield of 4 : 4′-dihydroxy-γ : δ-diphenyl-*n*-hexane (m.p. 184°) and a new synthesis from the reaction between anisaldazine and ethyl magnesium bromide yielded the methyl ether, melting at 144°, which on demethylation gave a small yield of the higher melting hexane derivative (m.p. 184°). The products of all syntheses possessed identical oestrogenic activity and it seems reasonable to suppose that this 4 : 4′-dihydroxy-

$\gamma:\delta$-diphenyl-*n*-hexane is the substance whose co-separation with impure anol caused that material to show surprisingly high activity. There were no indications whatever of the presence of any other oestrogen of even comparable potency in the anol by-products, although a careful examination was made of each fraction and sub-fraction.

A preliminary notice of the isolation of this substance from the anol by-products was made by Campbell, Dodds and Lawson (1938*b*) and an account of the first syntheses included in a paper by Dodds, Golberg, Lawson and Robinson (1939).

EXPERIMENTAL

Analysis of anol by-products

Anethole was heated for 40 hr. with two parts of potassium hydroxide and four parts of ethyl alcohol at 200°. The cooled reaction-mixture was steam-distilled to remove alcohol, diluted to 2 l./100 g. anethole and methylated by shaking with 150 g. methyl sulphate per 100 g. anethole, added in small quantities during 1 hr., the temperature being maintained at 35–40°. The mixture was steam-distilled to remove most of the reformed anethole, the residual thick oil distilled in glass apparatus at low pressure and two fractions collected: A, up to 150°/0·15–0·20 mm., which was then redistilled, and the residue added to B, which distilled between 160° and 190°/0·15–0·20 mm.

A was demethylated by heating with potassium hydroxide and alcohol in a sealed tube, and the resulting phenols fractionally crystallized. A fraction was obtained which yielded a 3 : 5-dinitrobenzoyl derivative melting at 116–117° (from ethyl alcohol). This did not depress the melting-point of *p*-*n*-propyl phenol 3 : 5-dinitrobenzoate (m.p. 118°). (Found: C, 58·1; H, 4·5. $C_{16}H_{14}O_6N_2$ requires C, 58·3; H, 4·3 %.)

B was redistilled, mainly passing over at 160–170°/0·15–0·20 mm. This fraction was dissolved in purified acetone (20 c.c./g.), cooled in ice, and finely powdered potassium permanganate, equal to the weight of the fraction, added. Stirring in an ice-bath was continued until reduction of the permanganate was complete. An equal weight of sodium bicarbonate was then added and the whole filtered and washed with acetone. The residue was extracted with boiling water and the aqueous liquid acidified. The precipitate consisted chiefly of anisic acid (m.p. 182°). The acetone solution was filtered and evaporated, and the oily residue distilled at low pressure. The first fraction (below 100°/0·3 mm.) was redistilled and was found to consist of α-*p*-methoxyphenyl *n*-propyl methyl ketone—identified by its semicarbazone (m.p. 189°). The combined residues were dissolved in acetone and the oxidation repeated, employing half of their weight of potassium permanganate, any excess after 40 hr. being removed by cautious addition of acetaldehyde to the warm liquid. The liquid was filtered, the acetone removed and the residue fractionated at low pressure. The fraction distilling at 120–145°/0·09 mm. deposited crystals after standing 2–3 weeks. They were collected and recrystallized from alcohol (m.p. 144°). (Found: C, 80·7; H, 8·8; OCH_3, 20·1. $C_{20}H_{26}O_2$ requires C, 80·5; H, 8·8; OCH_3, 20·8 %.)

The substance (94 mg.) was heated with 1 g. potassium hydroxide and 2 c.c. alcohol in a sealed tube at 200° for 18 hr. The contents of the tube were extracted with water, filtered and acidified; 50 mg. of a crude crystalline phenol were obtained. This was recrystallized twice from light petroleum (m.p. 184–185°). (Found: C, 79·9; H, 8·2. $C_{18}H_{22}O_2$ requires C, 80·0; H, 8·2 %.) The yield was of the order of 0·01–0·02 % on the anethole used initially.

The residual non-crystallizing portion of the oil-fraction (120–145°/0·09 mm.), after removal of its crystalline component, was heated with potassium hydroxide and alcohol at 200° and a brown gum produced, which partly crystallized in contact with benzene. The crystals were collected and recrystallized several times from benzene, m.p. 122° rising to 128° after further recrystallization from cyclohexane. (Found: C, 79·85; H, 8·2. $C_{18}H_{22}O_2$ requires C, 80·0; H, 8·2 %.)

1 : 4-*di*-(*p*-*hydroxyphenyl*)-2-*methyl*-1-*pentene*

"Isoanethole" (50 g.) (Goodall and Haworth 1930) was heated in an autoclave with potassium hydroxide (100 g.) and ethyl alcohol (200 c.c.) for 18 hr. at 200°. The mixture was cooled, extracted with water, steam-distilled to remove alcohol, cooled, acidified and extracted with ether. The ether solution was washed, dried and the solvent removed. The resulting gum was distilled under reduced pressure, the main fraction distilling at 274–277°/23 mm. 25 g. of this fraction were acetylated with acetic anhydride, and the acetyl derivative distilled. 22 g. distilled between 282 and 289°/25 mm. (Found: C, 75·0; H, 6·9. $C_{22}H_{24}O_4$ requires C, 75·0; H, 6·9 %.) The acetyl derivative (20 g.) was hydrolysed by boiling with a solution of potassium hydroxide (40 g.) in ethyl alcohol (400 c.c.) for 2 hr. The recovered phenol was distilled at reduced pressure and the main fraction (265–266·5°/20 mm.) redistilled at low pressure, collecting 184–185°/0·15 mm. (Found: C, 80·4; H, 7·6. $C_{18}H_{20}O_2$ requires C, 80·55; H, 7·5 %.)

1 : 4-*di*-(*p*-*methoxyphenyl*)-2-*methyl pentane*

"Isoanethole" (10 g.) was dissolved in acetone (100 c.c.) and hydrogenated in the presence of palladium catalyst (0·2 g.) at room temperature and pressure. 850 c.c. hydrogen were absorbed in 5 hr. The oily product was distilled at low pressure, b.p. 167°/0·08–0·09 mm. (Found: C, 80·5; H, 8·7. $C_{20}H_{26}O_2$ requires C, 80·5; H, 8·8 %.) Yield 7 g.

1 : 4-*di*-(*p*-*hydroxyphenyl*)-2-*methyl pentane*

The dimethyl ether (2 g.) was heated at 170° with potassium hydroxide (4 g.) and ethyl alcohol (8 c.c.) for 18 hr. The oily phenol crystallized on standing and was recrystallized several times from cyclohexane (m.p. 128°). (Found: C, 79·85; H, 8·2. $C_{18}H_{22}O_2$ requires C, 80·0; H, 8·2 %.)

1 : 4-*di*-(*p*-*methoxyphenyl*)-2 : 3-*dimethyl butan*-2 : 3-*diol*

p-Methoxyphenyl acetone (50 g.) (Goodall and Haworth 1930) was heated under reflux on a water-bath for 18 hr. with dry benzene (50 c.c.), coarse aluminium powder (5 g.) and mercuric chloride (2·5 g.). Water (25 c.c.) and benzene (25 c.c.) were then added and the mixture heated for a further hour, then filtered. The residue was boiled for 15 min. with benzene, filtered, and the combined filtrates freed from water and benzene. The residue was distilled to remove unchanged ketone (recovery 10 g.) and the gummy residue crystallized from ethyl acetate-light petroleum (b.p. 60–80°), then recrystallized from 80 % acetic acid and twice from alcohol (m.p. 135°). (Found: C, 72·7; H, 7·7. $C_{20}H_{26}O_4$ requires C, 72·7; H, 7·9 %.) Yield 10 g.

1 : 4-*di*-(*p*-*methoxyphenyl*)-2 : 3-*dimethyl butadiene*

The pinacol (6 g.) was heated under reflux with acetyl chloride (15 c.c.) and acetic anhydride (30 c.c.) for 2 hr. The mixture was cooled, poured into water, warmed to complete hydrolysis of reagents and cooled. The crystalline product was recrystallized from alcohol (m.p. 163–164°). (Found: C, 81·6; H, 7·7. $C_{20}H_{22}O_2$ requires C, 81·6; H, 7·5 %.) Yield 1·5 g.

1 : 4-*di*-(*p*-*methoxyphenyl*)-2 : 3-*dimethyl butane*

Di-(*p*-methoxyphenyl) dimethyl butadiene (1 g.) was hydrogenated in acetone solution in the presence of palladium catalyst (0·2 g.). The theoretical quantity of hydrogen was absorbed in 45 min. The product was recrystallized from alcohol (m.p. 82–83°). (Found: C, 80·5; H, 9·05. $C_{20}H_{26}O_2$ requires C, 80·5; H, 8·8 %.) Yield 0·7 g.

1 : 4-*di-(p-hydroxyphenyl)*-2 : 3-*dimethyl butane*

The dimethyl ether (0·2 g.) was heated for 2 hr. with acetic acid (2 c.c.) and hydriodic acid sp. gr. 1·94 (8 c.c.) at 140°. The mixture was diluted with water and the product crystallized from benzene-light petroleum (m.p. 151–152°). (Found: C, 79·6; H, 8·5. $C_{18}H_{22}O_2$ requires C, 80·0; H, 8·2 %.) Yield 0·1 g.

1 : 4-*di-(p-methoxyphenyl)*-1-*hexenone*-3

(*p*-Methoxyphenyl) *n*-propyl methyl ketone (12·7 g.) (Goodall and Haworth 1930) was mixed with anisaldehyde (9 g.) in alcohol (30 c.c.) and a few drops of alcoholic sodium ethoxide added. The mixture was boiled for 5 min. and cooled. The crystalline product was recrystallized from alcohol (m.p. 76). (Found: C, 77·3; H, 7·2. $C_{20}H_{22}O_3$ requires C, 77·4; H, 7·15 %.) Yield 14 g.

1 : 4-*di-(p-methoxyphenyl) hexan*-3-*one*

The unsaturated ketone (10 g.) was hydrogenated as above, absorbing the theoretical quantity of hydrogen in 2 hr. The product was recrystallized from alcohol (m.p. 69°). (Found: C, 77·0; H, 7·7. $C_{20}H_{24}O_3$ requires C, 76·9; H, 7·7 %.) Yield 9 g.

1 : 4-*di-(p-methoxyphenyl) hexane*

The ketone (8 g.) was boiled under reflux for 20 hr. with zinc wool (30 g.), previously amalgamated in 5 % mercuric chloride solution, fuming hydrochloric acid (50 c.c.) and acetic acid (50 c.c.). The mixture was cooled and diluted with water. The product was recrystallized from alcohol (m.p. 53°). (Found: C, 80·4; H, 8·7. $C_{20}H_{26}O_2$ requires C, 80·5; H, 8·8 %.) Yield 5 g.

1 : 4-*di-(p-hydroxyphenyl) hexane*

The dimethyl ether (4 g.) was boiled gently with hydriodic acid sp. gr. 1·7 (100 c.c.) for 2 hr. in a slow current of nitrogen. The product was worked up in the usual manner and crystallized in contact with light petroleum. It was recrystallized from light petroleum and from benzene (m.p. 98°). (Found: C, 79·8; H, 8·2. $C_{18}H_{20}O_2$ requires C, 80·0; H, 8·2 %.) Yield 2 g.

1 : 6-*di-(p-methoxyphenyl) hexandione* (1 : 6)

Adipic acid (25 g.) was converted into the chloride by gentle warming with thionyl chloride (200 c.c.) for 1 hr., after which excess thionyl chloride was removed at low temperature. The product was dissolved in carbon disulphide (100 c.c.) and added gradually to a cooled, stirred mixture of powdered aluminium chloride (60 g.), anisole (35 c.c.), and carbon disulphide (200 c.c.). Stirring was continued for 5 hr. and the mixture left overnight. The whole was poured into hydrochloric acid and crushed ice, freed from carbon disulphide and anisole by steam distillation, and the residual solid recrystallized twice from alcohol (m.p. 146°). (Found: C, 73·5; H, 6·8. $C_{20}H_{22}O_4$ requires C, 73·6, H, 6·8 %.) Yield 20 g.

1 : 6-*di-(p-methoxyphenyl) hexane*

See also v. d. Zanden 1938.

The diketone (10 g.) was reduced by Clemmensen's method, a little tetrahydronaphthalene being added to assist liquefaction. The product was recrystallized from light petroleum (m.p. 71°). Yield 4·8 g.

1 : 6-*di-(p-hydroxyphenyl) hexane*

The dimethyl ether (3 g.) was boiled gently with hydriodic acid sp. gr. 1·7 (75 c.c.) in a slow current of nitrogen for 2 hr. The crystalline product was recrystallized from benzene (m.p. 143–144°). (Found: C, 79·9; H, 8·2. $C_{18}H_{22}O_2$ requires C, 80·0, H, 8·2 %.) Yield 1·5 g.

New synthesis of 4 : 4′-*dihydroxy-γ* : *δ-diphenyl hexane*

See also Dodds, Golberg, Lawson and Robinson (1939).

Anisaldazine (13·4 g.) was slowly added to a Grignard solution prepared from magnesium (4·4 g.) and ethyl bromide (22 g.) in dry ether (150 c.c.). The mixture was heated under reflux for 1½ hr., cooled and decomposed with crushed ice and ammonium chloride. The ethereal layer was washed, dried and saturated with hydrogen chloride. After standing overnight in a refrigerator the ether was decanted, diluted with an equal volume of light petroleum, shaken and filtered. The filtrate was washed with water and with sodium bicarbonate solution, dried and the solvent removed. The residual oil deposited crystals on standing which were pressed on a porous title and recrystallized from alcohol (m.p. 144°). This was demethylated with potassium hydroxide and alcohol at 200° and the product crystallized from benzene (m.p. 184°). Yield 0·1 g.

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W. Schoeller of Schering-Kahlbaum A.G., Berlin, who kindly informed us of his early experiments demonstrating the presence of activity in the anol mother-liquor, and also Dr R. K. Callow and Mr V. H. Parker, of the National Institute for Medical Research, who investigated the absorption spectra of these substances.

SUMMARY

Demethylation of anethole by heating with potassium hydroxide and alcohol under pressure produces three oestrogenic substances of which one, produced in very small amount, is intensely active. These three have been isolated and identified, the most potent being 4 : 4′-dihydroxy-γ : δ-diphenyl-*n*-hexane. Syntheses of these and of several other dihydrodimerides of *p*-propenyl phenol are also described.

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The dynamic constants of human muscle

By A. V. Hill, Sec.R.S.

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In a recent paper (Hill 1938) the shortening of an isolated muscle against a constant force was shown to obey the characteristic equation

$$(P+a)(v+b) = \text{a constant} = (P_0+a)\,b,$$

where P is force, v is velocity of shortening, P_0 is the full isometric tension and a and b are constants. In frog's muscle a/P_0 was found to be about 4, and b (at 0° C) about one-third of the muscle length per second with a temperature coefficient of about 2 per 10° C. The theory was applied (Hill 1939*b*) in a study of the mechanical efficiency (work/total energy) of frog's muscle shortening at various speeds.

On human muscles there are not, to my knowledge, any experimental facts by which the characteristic equation can be directly tested and the constants determined. It is possible, however, to use the rather accurate results obtained with the inertia wheel (Hill 1922; Lupton 1922; Hill, Long and Lupton 1924) for an indirect test and an approximate calculation of the constants. The characteristic equation must then be modified as follows to meet the case of a constant mass instead of a constant load.

We make certain simplifying assumptions:

(i) That the maximum force which can be exerted by the arm on the string running from the hand to the pulley is constant ($=P_0$) at all angles of flexion of the arm. That this is approximately the case is shown by the curves of figure 3 in the paper by Hansen and Lindhard (1923).

(ii) That the line of the string remains sensibly parallel to the muscles of the arm (i.e. horizontal) throughout the shortening: this will be true if the string is long enough.

(iii) That as the muscles shorten the horizontal component of the movement of the hand is proportional to the amount of shortening of the muscles.

(iv) That the velocity of the hand and its constant b are *greater* than those of the muscles in that proportion; while

(v) The horizontal force exerted by the hand and its a are *less* than those of the muscles in the same proportion.

The muscles, the forearm and the string are supposed to conform to the simple diagram of figure 1. The reality is of course more complex, but impossible to express in manageable mathematical form. We will assume therefore that in the characteristic equation P, P_0 and a are $1/n$ of those of the muscles; while v and b are n times those of the muscles. Thus the identical equation applies and the constant is the same for the arm pulling the string as for the muscles.

Let x be the horizontal distance through which the string is pulled. Then if M be the "equivalent mass" of the wheel (see Hill 1922),

$$M\ddot{x} = P,$$

which, from the characteristic equation (since $\dot{x} = v$), is

$$M\ddot{x} = (P_0 + a)\,b/(\dot{x} + b) - a.$$

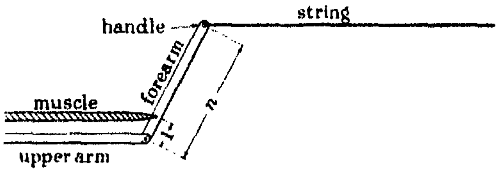

FIGURE 1. Simplified diagram of arm.

Multiply by $(\dot{x} + b)\,dt$ and integrate from 0 to t,

$$\tfrac{1}{2}M\dot{x}^2 + bM\dot{x} + ax = P_0 bt. \tag{1}$$

Multiply by $(\dot{x} + b)$ and rearrange,

$$\frac{d\dot{x}}{b}\left[-1 + \frac{(P_0/a + 1)}{P_0/a - \dot{x}/b}\right] = \frac{a\,dt}{Mb}.$$

Integrate from 0 to t,

$$-\dot{x}/b - (P_0/a + 1)\log_e\left(1 - \frac{\dot{x}}{b}\frac{a}{P_0}\right) = \frac{at}{Mb}. \tag{2}$$

From (1) and (2) eliminating t, writing c for P_0/a and u for $\dot{x}/bc$,

$$\frac{1}{M}\frac{ax}{(bc)^2} = -\left(1 + \frac{1}{c}\right)\log_e(1 - u) - \left(1 + \frac{1}{c}\right)u - \tfrac{1}{2}u^2. \tag{3}$$

In this equation, relating the final velocity to the mass pulled, the velocity u is expressed as a fraction of the maximum velocity bc of the unloaded muscle.

The work done on the wheel is $\tfrac{1}{2}M\dot{x}^2$, the maximum work which would be done (disregarding fatigue) at very low speed is $P_0 x$. From this the work W can be expressed as a fraction w of the maximum work W_0 as follows:

$$w = \frac{u^2/2c}{-(1 + 1/c)\log_e(1 - u) - (1 + 1/c)u - \tfrac{1}{2}u^2}. \tag{4}$$

From (2) and (3) the time for shortening the distance x is

$$t = \frac{x}{bc}\,\frac{(1+1/c)\log_e(1-u)+u}{(1+1/c)\log_e(1-u)+(1+1/c)\,u+\frac{1}{2}u^2}. \qquad (5)$$

The numbers in table 1 are calculated from equations (3), (4) and (5) for $c = P_0/a = 4$, the mean value found by Hill (1938) for frog's muscle. Similar calculations have been made for $c = 2$, 3 and 5. It is desired to fit the calculation to the experimental data. For this purpose w is plotted against $\log M$ and the distance by which the theoretical curve has to be shifted to make it coincide with the observed points gives $\log ax/b^2$.

TABLE 1. CALCULATED FROM EQUATIONS (3), (4) AND (5) FOR $P_0/a = c = 4$

$u = v/bc =$	1·0	0·995	0·9875	0·975	0·95	0·925	0·875	0·825
$M = ax/b^2 \times$	0	0·01278	0·01664	0·02143	0·02968	0·03779	0·05567	0·07743
$w = W/W_{\text{max.}} =$	0	0·02534	0·03245	0·04074	0·05357	0·06467	0·08524	0·1045
$t = x/b \times$	0·25	0·2881	0·2989	0·3116	0·3318	0·3496	0·3840	0·4193
$u = v/bc =$	0·75	0·625	0·5	0·375	0·25	0·2125	0·175	0·15
$M = ax/b^2 \times$	0·1216	0·2505	0·5368	1·290	3·942	6·003	9·762	14·21
$w = W/W_{\text{max.}} =$	0·1368	0·1957	0·2684	0·3629	0·4928	0·5421	0·5979	0·6394
$t = x/b \times$	0·4779	0·6023	0·7868	1·097	1·728	2·068	2·556	3·021
$u = v/bc =$	0·125	0·1	0·075	0·0625	0·05	0·0375	0·025	0·0125
$M = ax/b^2 \times$	21·92	36·75	70·26	105·1	170·5	314·9	737	3069
$w = W/W_{\text{max.}} =$	0·6849	0·7350	0·7905	0·8207	0·8523	0·8855	0·9212	0·9592
$t = x/b \times$	3·674	4·660	6·310	7·636	9·626	12·94	19·60	39·57

The best experimental results relating work W to mass M are those of Lupton (1922, tables 2 and 4, mean of 9 experiments). These, as corrected for the slight effect of fatigue in the longer pulls by Hill *et al.* (1924), are given in table 2(*a*). Lupton records that the observed mean length of pull was 61 cm.: x therefore has been taken as the round figure of 60 cm.

For greater accuracy a correction is necessary for the equivalent mass and the kinetic energy of the forearm of the subject. The original subject is not at present available, but my own arm must be so similar to his that no important error can result from using measurements with mine. It is shown below that the moment of inertia of the forearm must have added about 0·45 kg. to the equivalent mass, and so the total kinetic energy must have been greater than that of the wheel alone by a fraction $0{\cdot}45/M$ of the latter. These corrections have been applied in table 2(*b*).

Over the range of equivalent masses examined by Hill and by Lupton a sufficiently close agreement was found with the formula

$$W/W_0 = 1 - k/t,$$

where W_0 was a constant supposed to represent the maximum work and k another constant supposed to represent the "viscosity" of the muscle. The simple "viscosity" theory is now known (Hill 1938) not to be valid, and Hansen and Lindhard already in 1923 had found an inconsistency in the relation. According to them the value of the maximum work deduced from a length-tension diagram, made by measuring the maximum force capable of being exerted at each stage of a pull, is considerably greater than W_0 obtained by fitting the formula $W/W_0 = 1 - k/t$ to the uncorrected results with the wheel. In the two experiments quoted by Hansen and Lindhard the excess is roughly 15 % (Hill; Appendix to Lupton 1923). The divergence of the experimental points from the curve $W/W_0 = 1 - k/t$ clearly occurs only at very low speeds, i.e. at very large masses beyond the range of the wheel.

TABLE 2

(a) *Experimental relation* (*Lupton* 1922, *tables* 2 *and* 4, *mean of* 9 *experiments*) *between work and equivalent mass corrected for the effect of fatigue*

M, kg.	681	348	208	70·8	36·94	22·69	14·04	11·67
W, kg.m.	10·51	9·76	9·15	7·72	6·67	5·76	4·95	4·66
t, sec.	2·16	1·60	1·25	0·83	0·66	0·55	0·51	0·46

(b) *Results of* (*a*) *further corrected for the mass of the arm*

M, kg.	681·4	348·4	208·4	71·25	37·39	23·14	14·49	12·12
W, kg.m.	10·51	9·77	9·17	7·77	6·75	5·87	5·11	4·84
$\sqrt{M}$	26·10	18·66	14·43	8·44	6·11	4·81	3·81	3·48
$w = W/13$	0·809	0·751	0·705	0·597	0·520	0·452	0·393	0·372

(c) *Experimental relation* (*Lupton* 1922, *table* 5, *mean of* 8 *experiments*) *between work and mass, corrected as above for* (i) *the effects of fatigue, and* (ii) *the mass of the arm*

M, kg.	681·4	348·4	208·4	71·25	37·39	23·14	14·49	12·12
W, kg.m.	10·10	9·33	8·77	7·50	6·43	5·73	5·21	4·90
$\sqrt{M} \times 1·02$	26·52	19·03	14·72	8·61	6·23	4·91	3·89	3·55
$w = W/12·5$	0·808	0·746	0·701	0·600	0·514	0·459	0·417	0·392
t, sec.	2·09	1·54	1·25	0·79	0·62	0·52	0·45	0·42

The value of W_0 calculated by Lupton from his uncorrected results was 10·96 kg.m. We will take 13·0 kg.m., a round figure 18·6 % greater, in agreement with Hansen and Lindhard. The distance pulled x being 60 cm. and the maximum work being $P_0 x$, $P_0 x = 13·0$ and $P_0 = 21·67$ kg. wt. Thus of the four constants available for fitting the experimental results of table 2(*b*) to the theoretical relation (table 1) between w and M, three have been chosen on independent evidence:

(i) $P_0 = 21{\cdot}67$ kg. wt. $= 21{\cdot}67g$ absolute units;
(ii) $a = P_0/4 = 5{\cdot}417$ kg. wt. $= 5{\cdot}417g$ absolute units;
(iii) $x = 60$ cm.

We are left therefore with b available to help us to fit the equations. Some independent evidence of the value of b is given below, from the maximum speed of the unloaded arm. This, however, is not sufficiently exact for calculation, and is confirmatory only; and in table 3 from which figure 2 is constructed b is taken as 202·6 cm./sec. Thus $ax/b^2 = 7{\cdot}77$, $x/b = 0{\cdot}2961$ and the constant of the characteristic equation is $5487g$ absolute units (kg. cm. sec.).

TABLE 3. GENERAL RESULTS OF TABLE 1 CALCULATED FOR THE SPECIAL VALUES: $a = 5{\cdot}417 \times g$ KILODYNES; $b = 202{\cdot}6$ CM./SEC.; $x = 60$ CM.; $c = P_0/a = 4$

v, cm./sec.	810	806	800	790	770	750	709	669
M, kg.	0	0·0994	0·1293	0·1665	0·2306	0·2936	0·4326	0·6017
$w = W/W_0$	0	0·0253	0·0325	0·0407	0·0536	0·0647	0·0852	0·1045
t, sec.	0·0740	0·0853	0·0885	0·0923	0·0983	0·1035	0·1137	0·1242
$\sqrt{M}$	0	0·315	0·360	0·408	0·480	0·542	0·658	0·776

v, cm./sec.	608	506	405	304	203	172	142	122
M, kg.	0·9446	1·947	4·171	10·025	30·63	46·64	75·85	110·4
$w = W/W_0$	0·1368	0·1957	0·2684	0·3629	0·4928	0·5421	0·5979	0·6394
t, sec.	0·1415	0·1784	0·2330	0·3248	0·5119	0·6123	0·7570	0·8946
$\sqrt{M}$	0·972	1·396	2·042	3·163	5·535	6·830	8·710	10·51

v, cm./sec.	101·3	81·0	60·8	50·6	40·5	30·4	20·3	10·1
M, kg.	170·3	285·6	546·0	816·3	1325	2446	5726	23846
$w = W/W_0$	0·6849	0·7350	0·7905	0·8207	0·8523	0·8855	0·9212	0·9592
t, sec.	1·0881	1·380	1·869	2·261	2·851	3·833	5·803	11·72
$\sqrt{M}$	13·05	16·90	23·36	28·57	36·38	49·46	75·67	154·4

In the upper curve of figure 2 w is plotted against $\sqrt{M}$ from table 3: the observed points (table 2(b)) are given as hollow circles. The agreement is satisfactory. It is clear that by choosing a suitable value of b a close fit can be obtained. The chief purpose of using $\sqrt{M}$ instead of M is to show the curve in more manageable form, to expand the scale at small and to contract it at great values.

In figure 2 the solid circles represent the results of table 2(c), the mean of eight experiments by Lupton on another subject, similarly corrected for fatigue and for the mass of the forearm. With $P_0/a = 4$, identically the same value of b is required. For this subject W_0 calculated from the equation $W/W_0 = 1 - k/t$ was 10·54 kg.m., so the maximum work P_0x has been taken as 12·5 kg.m. which is 18·5 % greater. In the other subject it was 13·0 kg.m.

Since x was the same as in the other subject (60 cm.), P_0, and therefore a, is 4 % less in the second subject than in the first. For a given final velocity equation (3) shows that M varies as a. Thus the theoretical curve for the second subject should have been plotted against a value of M which is 4 %, or a value of $\sqrt{M}$ which is 2 %, less than for the first subject. To avoid plotting two curves it was simpler to multiply the observed $\sqrt{M}$ by 1·02 before plotting: this has the same effect and avoids overlapping between the plots of the two experiments. We see that the second experiment fits the theoretical curve even better than the first one.

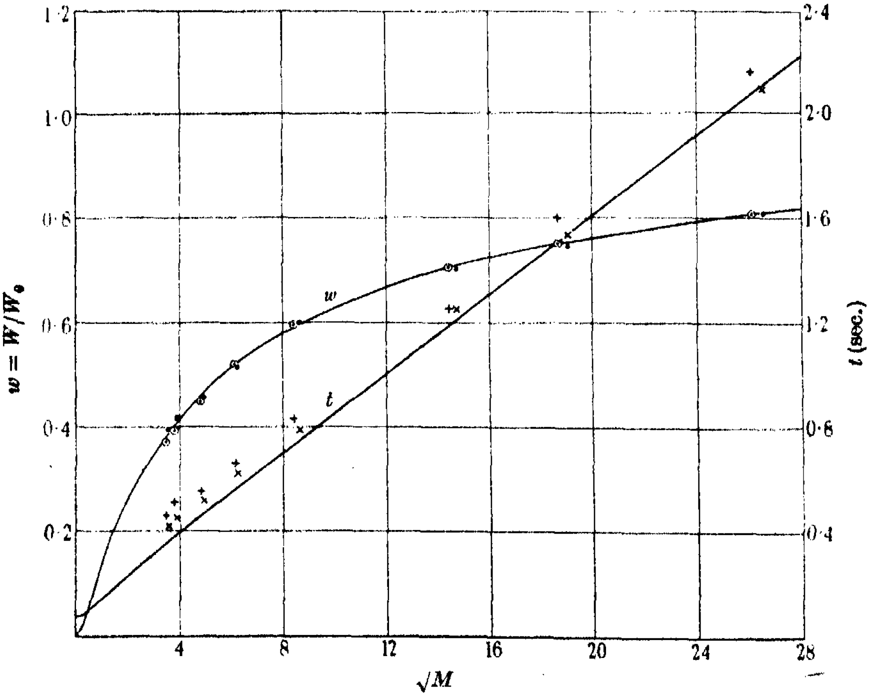

FIGURE 2

Curve w, theoretical relation (table 3) between relative work $w = W/W_0$ and mass pulled. Hollow circles, table 2(*b*). Solid circles, table 2(*c*). Scale on left.

Curve t, theoretical relation between time and mass. +, table 2(*b*). ×, table 2(*c*). Scale on right.

The other curve of figure 2, which is very nearly a straight line, represents t plotted against $\sqrt{M}$ (table 3) and the crosses are the observed times (table 2(*b*) +; table 2(*c*) ×). A curious fact emerges, viz. that all the observed points in the first experiment (+) are too high and by nearly the same amount, 0·09 sec.: in the second experiment (×) all but the last two points are too high by a mean amount 0·05 sec. It seems most likely that in

Lupton's experiments the method of measuring the times had a consistent error, making them 0·09 sec. too long for the first subject, 0·05 sec. too long for the second. The beginning of the movement occurred at the quick release of the wheel which opened a short circuit of the magnet recording the time: at the end of the movement the arm of the subject knocked down a lever which short-circuited the magnet again. The closing of the short circuit at the end may have occupied a finite time depending on the manner in which the arm of the subject hit the lever.

The relation between w and $\sqrt{M}$ in figure 2 shows very well the effect noted by Hansen and Lindhard, viz. that the maximum work can be obtained only by pulling enormous masses, altogether beyond the practical range: and the time occupied would be so great that fatigue would set in and considerably affect the result. With an equivalent mass of 500 kg. (half a ton) the work is still about 22 % short of the maximum. The reason is that the relation between velocity of shortening and force exerted (see Hill 1938, figure 12, p. 177) comes down very slowly to the force axis, so that even at very low velocities the force exerted is appreciably less than the maximum. The "viscosity" theory of muscle gives no explanation of this.

The moment of inertia of the forearm. The moment of inertia was found by timing the free oscillation of the forearm hanging vertically, the upper arm resting horizontally on the corner of a table with the front side downwards. Let M be the mass of the forearm, Mk^2 its moment of inertia, h the distance of its centre of gravity from the joint, and T the period of its free oscillation. Then

$$T = 2\pi\sqrt{(Mk^2/Mhg)}.$$

With a little practice the arm could be maintained in a gentle free oscillation without interfering with its natural period, as is shown by the very consistent results. The time for 20 oscillations was taken by a stop watch reading to 0·02 sec., and the following values were obtained for T:

27 Nov. Mean of 10 readings, 0·838 sec.: average difference from mean 0·011 sec.
3 Dec. Mean of 20 readings, 0·797 sec.: average difference from mean 0·0107 sec.

A weighted mean is 0·811 sec.

It is now necessary to know Mh. A pencil was held in the closed hand, in the same attitude as the handle used with the inertia wheel, and its point pressed against a plane surface. The forearm was revolved and a circle traced on the surface. The radius l of this circle was measured and a number of consistent measurements gave a mean of 35 cm. With the upper arm supported vertically, the forearm was now weighed: it rested freely and horizontally on a balance, the load being taken by a match box or other suitable support placed immediately below the pencil referred to above. If m be the balancing weight in the pan, taking moments about the joint,

$$ml = Mh.$$

For my arm m was 0·483 kg. and l was 35 cm. Thus $Mh = 16·9$ kg. × cm. Using these values and $T = 0·811$ sec., the formula gives $Mk^2 = 277$ kg. cm.^{2}

If the arm is revolving with velocity v at the handle, distant l from the joint, its kinetic energy is $\frac{1}{2}Mk^2(v/l)^2 = \frac{1}{2}(Mk^2/l^2)v^2$. Its "equivalent mass" at the handle is Mk^2/l^2. In the above example the equivalent mass is $277/35^2 = 0{\cdot}226$ kg. With the inertia wheel, however, the pull finished with the arm flexed through 135°. The pull, therefore, was $35 + 35/\surd 2 = 60$ cm. If the velocity of the string off the pulley, i.e. the horizontal velocity of the handle, was v, the total velocity of the handle was $v\surd 2$, so that the kinetic energy would be $\frac{1}{2}0{\cdot}226(v\surd 2)^2 = \frac{1}{2}0{\cdot}45v^2$. Thus, for the whole pull, the kinetic energy of the arm itself can be allowed for by assuming it to have an equivalent mass of 0·45 kg., which must be added on to that of the wheel.

The maximum velocity of the arm. According to the characteristic equation the unloaded muscle would shorten with velocity P_0b/a; which if $P_0/a = 4$ is $4b$, or in the present instance 810 cm./sec. It is not possible, however, even apart from friction in joint and tissues, to unload the muscle completely, because of the mass of the limb itself: an approximate allowance, however, can be made for the latter.

A simple method was used to find the maximum unloaded velocity, viz. to see how high a small mass attached by a string to the handle would rise when the arm was suddenly flexed as with the wheel. The upper arm was supported horizontally, biceps upwards (see Hill 1922, figure 1, p. 22) and a string went horizontally to a small light pulley some distance away in a wall. From there the string went over a pulley in the ceiling, and then dropped to near the ground. At the end of the string was a small weight, and behind it a vertical scale marked clearly on a board with white paint.

First the arm was bent slowly to its full extent, and the position of the weight noted. Then it was flexed as rapidly as possible and the maximum height above that position to which the weight was thrown was read on the scale. Observation was very easy: the only difficulty, and that was chiefly subjective, was to ensure that, with these very quick movements, no other movement than a pure flexion of the arm occurred; *and yet* that no restraint was exerted by antagonist muscles. With practice very consistent readings were obtained as the following results show. The pulleys were small and light and the string light, to avoid any effect of the kinetic energy of these in carrying the weight too high.

3 *Dec.* (*a*) Weight 135 g. 15 readings: mean height of throw 147 cm.: average difference from mean 8 cm. (*b*) Weight 341 g. 15 readings: mean height of throw 112·7 cm.: average difference from mean 8 cm.

8 *Dec.* (*a*) Weight 128 g. 25 readings: mean height of throw 147 cm.: average difference from mean 9·6 cm. (*b*) Weight 346 g. 25 readings: mean height of throw 107 cm.: average difference from mean 8·6 cm.

Even with these small weights the height of throw is appreciably less with the larger weight—as it should be according to theory. It is not practicable experimentally to use a very small weight (e.g. a few grams), since the string interferes with it too much during the throw. It is better therefore to use two slightly larger weights, and to extrapolate back linearly to zero weight. If this be done the first experiment gives 169·5 cm., the second 170·5 cm., mean 170 cm., as the height of throw of a very small weight. This corresponds to a velocity $v = \sqrt{(2g \times 170)} = 577$ cm./sec.

This is the maximum velocity parallel to the active muscles with which the hand can move at a distance 35 cm. from the elbow joint.

Only a rough correction can be made for the mass of the forearm itself, to find what effect it has on the maximum velocity. The equivalent mass of the arm being 0·45 kg. and the handle and string being 0·03 kg., the total is 0·48 kg. The subject of the present experiments is not now as strong as the subject of Lupton's in 1923. The previous subject would probably have given the same final velocity to a mass about 4/3 times as great, i.e. to 0·64 kg. If v in table 3 is interpolated for $M = 0{\cdot}64$ kg., we find $v = 660$ cm./sec. This is rather higher than the observed value 577 cm./sec.: considering, however, the number of assumptions and approximations made in the calculation, the agreement is not unsatisfactory. It is clear that the maximum velocity is of the order of size predicted by the theory.

The mechanical efficiency. According to Hill (1939*b*, p. 436) the maximum efficiency is determined by two constants, a/P_0 and K/P_0, where $K = k/b$ and k represents the energy required per second to maintain a contraction. For a maximum efficiency of 25 % over the whole oxidative cycle, the maximum initial efficiency is 50 %, assuming that the ratio of total energy to initial energy is 2 as in frog's muscle (Hill 1939*a*). If $a/P_0 = \frac{1}{4}$, a 50 % maximum efficiency would require that $K/P_0 = 0{\cdot}17$ (Hill 1939*b*, equation (6)), and would be attained at a speed of shortening $v/b = 0{\cdot}585$ (equation (5)). For $b = 202{\cdot}6$ cm./sec., therefore, the optimum speed would be 120 cm./sec. Thus the most efficient time for completing a single flexion of the arm of maximum extent (60 cm.) would be 0·5 sec., for a movement of smaller extent correspondingly less. According to Lupton (1923) the optimum duration of a single step up a staircase is about 1 sec., and Dickinson (1929) found about the same optimum duration of a single leg movement on a bicycle ergometer. It would seem very likely on general dimensional reasoning that the optimum speed of the muscles of a small limb like the arm would have been adjusted in a well-designed animal to be greater than that of a large limb like the leg (cf. Furusawa, Hill and Parkinson 1927). We should expect b to be less, when expressed as a fraction of the muscle's length or of its amplitude of movement, in the muscles of a large limb or of a large animal than in those of a small. Cooper and Eccles (1930) certainly found in the cat that the extensor digitorum longus and the internal rectus of the eye contracted much more quickly than the soleus and gastrocnemius.

The absolute value of P_0/a. The good fit of the curve to the observed points in figure 2 does not prove the correctness of the assumption that $P_0/a = 4$, as in frog's muscle. If we had taken $P_0/a = 5$ instead of 4, very nearly as good a fit would have been obtained with $b = 190$ cm./sec.: with $P_0/a = 3$, the fit would have been quite as good with $b = 218$ cm./sec. To find P_0/a experimentally it is necessary to find P_0 (which is easy) and to vary the speed of shortening over the widest possible range. This cannot be done with the wheel: it is best done in isotonic contractions with widely different loads.

It is unlikely, however, that P_0/a is far different from 4. The maximum speed of shortening (unloaded, and neglecting the mass of the arm) is P_0b/a. For $P_0/a = 3$ this would be 654 cm./sec., which is probably a little too low, seeing that the observed value, not neglecting the mass of the arm, is 577 cm./sec. For $P_0/a = 5$ it would be 950 cm./sec., which is almost certainly too high. So far therefore as this evidence goes the most likely value of P_0/a would seem to be between 3 and 4.

The absolute value of b. It seems fairly certain that the absolute value of b for the muscles of the human arm is of the order of 200 cm./sec., reckoned of course at the hand and not at the insertion of the muscles themselves. It was pointed out above that for $P_0/a = 5$ and 3 good fits can be obtained with $b = 190$ and 218 cm./sec. respectively. Even for $P_0/a = 2$ a good fit is found for $b = 218$ cm./sec., if W_0 be taken as 12·7 kg.m. instead of 13.

In frog's muscle at 0° C the value of b is about one-third of the muscle's length per second, increasing about twofold for a rise of temperature of 10° C. The time for a complete flexion of the arm (60 cm.) at speed $b = 200$ cm./sec. is 0·3 sec. If in such a flexion the muscles shorten three-tenths of their length, the value of b is muscle length per second, about the same as in a frog's sartorius at 16° C.

It would be very interesting to determine the value of b in other human muscles in terms of muscle length per second.

The possibility of reflex inhibition during rapid movement. In 1931 Fenn, Brody and Petrilli published an account of experiments on the relation in man between muscular force and speed of shortening. The force exerted when the limb was held fast was measured, and the acceleration was determined after a quick release. The acceleration measured the force exerted, and so the force was determined as the velocity increased. Fenn found a considerably smaller coefficient of "viscosity" than did Hill and Lupton in their experiments, i.e. less effect of velocity on the force exerted. He states that the maximum duration of the period of measurement was 0·016 sec. The object of confining the observations to so short a time was to avoid the possibility of reflex inhibition setting in and affecting the force exerted by the muscles. The possibility of such reflex inhibition had already been suggested by Hill (Lupton 1923, Appendix, p. 353) as an explanation of the fact that W_0 calculated from the equation $W/W_0 = 1-k/t$ was less than the maximum work determined by Hansen and Lindhard.

Fenn concluded that the falling off of force with increase of speed in Hill's and Lupton's experiments was too great, and suggested that this might be due to the reflex inhibition which he had avoided.

Now a muscle (Hill 1938) consists of two components in series, a contractile and an elastic one. During an isometric contraction the former stretches the latter: on quick release the latter shortens rapidly without sensible "viscosity", the former more slowly according to the characteristic equation. In the very early stages therefore, as in Fenn's experiments, the acceleration is determined largely by the properties of the elastic component and not by those of the contractile muscle. The process is very clearly illustrated in the curves given by Hill (1939*b*, figure 3, p. 441) for the tension exerted as a function of length at various speeds of shortening. At the higher speeds the drop in tension on release occurs gradually, not instantly as it would with the contractile component alone, until it reaches a value characteristic of the speed. Only when the shortening of the elastic element is complete does the characteristic force of the muscle at that speed occur.

Thus Fenn's experiments, it is seen now, provide no evidence on the relation between force and speed in the contractile component of the muscle. His objection, therefore, to the greater effect of speed on force found in Hill's and Lupton's experiments does not hold. The difficulty raised by Hansen and Lindhard has been resolved by applying the characteristic equation instead of the "viscosity" theory. There seems, therefore, to be no reason for not accepting the values obtained with the wheel as truly representative of muscle contracting maximally at various speeds.

CONCLUSION

It is very desirable that the simple relation between velocity v and force P in isotonic contraction of human muscle should be determined directly with adequate apparatus over a wide enough range of force and speed. This would allow a proper measurement to be made of the dynamic constants a, b and a/P_0 in various muscles. If, further, a direct determination could be made of the other constant $K = k/b$, representing the cost of maintaining a contraction, it would be most valuable. Is k approximately equal to ab as it is in frog's muscle? There is no possibility at present of my being able to undertake these measurements. The approximate calculations, therefore, and the simple experiments described above are published now, to show that the theory can account adequately for such facts as are known, for human muscle, on the relation between force and speed, and in the hope that perhaps others may be able to make the further experiments proposed.

SUMMARY

The characteristic equation of muscle is modified to meet the case of a constant mass accelerated instead of a constant force overcome at uniform speed.

The theory is compared with experimental results obtained on human arm muscles with an inertia wheel. The agreement is good.

The value of the dynamic constant b is deduced. Its order of size is muscle length per second. P_0/a cannot be very far from 4, its mean value in frog's muscle.

The maximum speed of shortening (unloaded) is determined experimentally. This is of the order of size $P_0 b/a$ deduced from theory.

The mechanical efficiency, the optimum speed, the possibility of reflex inhibition during rapid shortening and some proposals for further research are discussed.

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γ-Carotene in the sexual phase of the aquatic fungus *Allomyces*

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[Plate 16]

INTRODUCTION

It has long been noted that, among the majority of plants and animals, relatively concentrated stores of carotenoids are to be found in certain particular tissues, structures and secretions associated with reproduction. Many of the yellow, orange or red pigments of pollen, stamens, stigmata, petals, seeds, and fleshy parts of fruits of higher plants are outstanding examples. Likewise, among animals, considerable attention has been given to the striking manifestation of similar pigments in the eggs of countless species of invertebrates and fishes, as well as in those of birds and some amphibians and reptiles, and in the corpora lutea and milk-fat of certain mammals. Investigations of such compounds (even apart from the A vitamins and their common precursors) in reproductive structures and their accessories have suggested to many the possibility that these pigments play definite biochemical roles in processes associated with sexual maturation, fertilization, and embryonic development.‡

Although the carotenoids are not as universally distributed among the fungi as they are in other cryptogams, pigments of this type have been identified in a wide variety of Basidiomycetes and Ascomycetes, as well as in some genera of Myxomycetes and Phycomycetes. As far as we are aware, however, the concentration of carotenoids in the *sexual* reproductive structures of fungi is comparatively rare. Of particular interest in this connexion are the studies of pigments in the Mucorales. Lendner (1918),

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† Scripps Institution of Oceanography of the University of California, La Jolla, California. At Cambridge 1938–9.

‡ For detailed reviews, relating to the occurrence and possible significance of carotenoids, reference may be made to the extensive writings of Zechmeister (1934, 1937), Lederer (1934, 1935, 1938), and Strain (1938), as well as to the earlier work of Palmer (1922) and of Verne (1926).

and Satina and Blakeslee (1926*a*) remarked upon the occurrence of a yellow substance, concentrated particularly in the progametes and gametes of many Mucors. In 1927 Chodat and Schopfer, after extracting and testing the pigment from *Mucor hiemalis*, identified it as a carotene. Their data suggest that α-carotene may have been the preponderant pigment, mixed, perhaps, with the β-isomer.

In the group of Phycomycetes known as the water-moulds, methods of reproduction are very varied and of unusual interest. Orange-coloured pigments are only rarely formed in obvious quantities within the cytoplasm of these aquatic fungi, but the few known examples of such pigmentation are striking. Some Chytridiales, notably certain species of the genus *Synchytrium*, are characterized by the presence of an orange-red substance which is contained in their sporangia and spores. Two other genera of water-moulds, *Blastocladiella* and *Allomyces*, also synthesize and store orange pigment, the occurrence of which is made singularly interesting by the fact that in most species of these fungi it is confined entirely to the sexual cells, and, moreover, to cells of one sex only.

Hitherto, as far as we know, no one of the cytoplasmic pigments in the water fungi has been investigated in detail. Kniep (1929) stated that the colour of the male gametangia in *Allomyces* depends upon a carotene ("...auf einem Karotin beruht", p. 204), but he presented no evidence to support this statement and made no further mention of it elsewhere in his two papers on *Allomyces*. The manifestation of profound pigmentary differences between the sexual and asexual generations of *Allomyces* suggested that a preliminary study (by D. L. F.) of the chemical nature of the pigments involved, combined with certain features of the current investigations (by R. E.) of sexuality and inheritance in the water-moulds, particularly *Allomyces*, would be of value. The results of such an investigation are reported in the present paper.

BIOLOGY OF *ALLOMYCES*

Before discussing the chemistry of the pigments, it will be well to review briefly the main points of interest in the morphology and life history of the fungus. In water culture, growing on natural substrata such as dead insects, seeds, plant debris, etc., *Allomyces* forms a characteristic growth similar in appearance to that of the more common water-moulds *Achlya* and *Saprolegnia*. The main part of the plant-body is composed of dichotomously branching filaments, which are one to two centimetres long and bear the reproductive organs at their tips.

The interesting type of sexual reproduction and the morphological alternation of generations exhibited in the life-cycle of certain species of *Allomyces* were first described by Kniep in 1929 and 1930. Since that time Hatch (1933), Sörgel (1937) and others have verified and extended Kniep's discoveries, and only a brief account of the significant stages in the life history need be given here. As shown in figure 1, an asexual or sporophytic generation of plants (E) regularly alternates with a sexual or gametophytic generation (B). Asexual plants bear two kinds of sporangia, thin-walled zoosporangia and heavy-walled resistant sporangia. Spores (not shown in figure 1) from the former give rise to further sporophytes directly, while those from the latter develop into gametophytes (B). These sexual plants

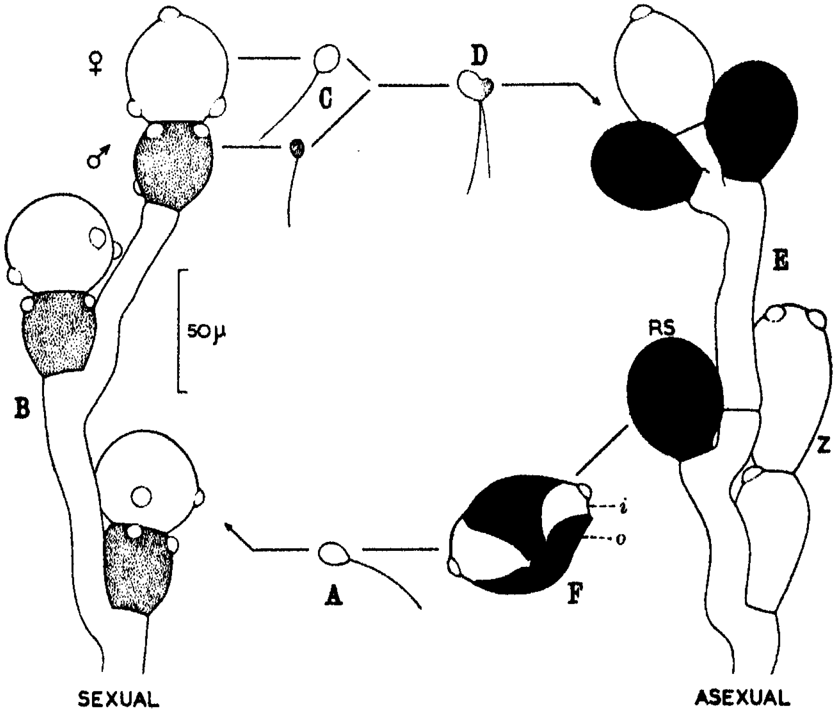

FIGURE 1. Life cycle of *Allomyces* (subgenus *Euallomyces*) showing: A, zoospore from resistant sporangium; B, gametophytic hypha bearing female (♀) and male (♂) gametangia; C, female and male gametes; D, planozygote; E, sporophytic hypha bearing zoosporangia (*Z*) and resistant sporangia (*RS*); F, germinating resistant sporangium with outer wall (*o*) and inner membrane (*i*). Semidiagrammatic. Approximately × 335 as here reproduced. The outer walls of resistant sporangia are shown in black to represent melanin pigment. Stippled structures contain carotene within the cytoplasm. All structures shown merely in outline are unpigmented.

are monoecious and usually bear male and female gametangia in pairs. The female gametangia are somewhat larger than the males and, in the primary pairs, are regularly borne either terminally, as in *A. arbuscula*, or subterminally, as in *A. javanicus*. In addition to these differences between the size and position of female and male gametangia, there is a marked colour difference which will be described in detail below. The gametes (C) which emerge show a similar colour difference, and the diameter of the females in more than twice that of the males. Both types are motile, however, and nearly identical in structure. They fuse in pairs to produce biflagellate zygotes (D), which subsequently germinate, form sporophyte plants and thus complete the cycle.

Bearing this life history in mind we may turn to a consideration of the conspicuous pigments which occur in *Allomyces*. Sporophyte plants, whether growing in water or on agar, have a very characteristic brown colour, imparted to them by pigment(s) in the heavy, pitted, outer walls of the mature resistant sporangia. The inner membrane and the cytoplasm of these sporangia, as well as the spores which are produced, are colourless. Indeed, with the exception of the outer walls of the resistant sporangia, all parts of the asexual plants are without colour; no visible traces of pigment are to be found anywhere within the cytoplasm. This condition is in marked contrast to that which occurs in the gametophyte: here, while hyphae and female gametangia are quite unpigmented, the male gametangia are coloured a brilliant orange. The pigment is contained in oil droplets or lipoid granules within the cytoplasm. It is not found in the walls of the male gametangia which are perfectly hyaline and colourless after liberation of the gametes. The colour is still clearly visible in the male gametes after their emergence, and is frequently to be seen even in the zygotes shortly after plasmogamy has taken place. Then, during the short quiescent stage of the zygotes, but preceding germination, the pigment disappears, and no traces of it can be seen at any time in the life cycle until it reappears in the male gametangia of the next gametophyte generation. Although male and female gametangia are borne directly adjacent to each other on the same hyphae, those cells which give rise to the large female gametes have never been observed to show any traces of yellow pigment.

The life history which we have just outlined, characterized by an obvious alternation of asexual and sexual generations and a fusion of anisogametes, is not the only one which is known in *Allomyces*. Emerson (1938) has shown that *A. moniliformis* and similar forms have a markedly different cycle with only one generation of plants, which, like the sporophytes of the previously described cycle, bear resistant sporangia and thin-walled

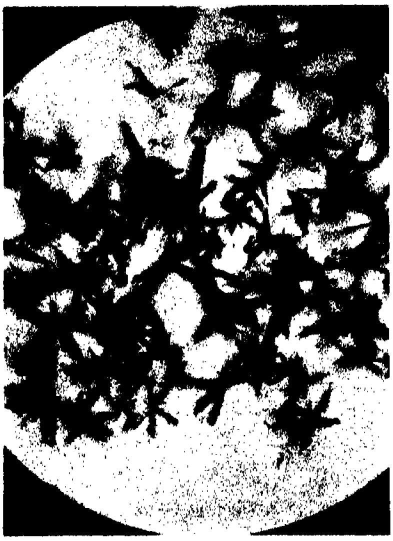

Figure 2

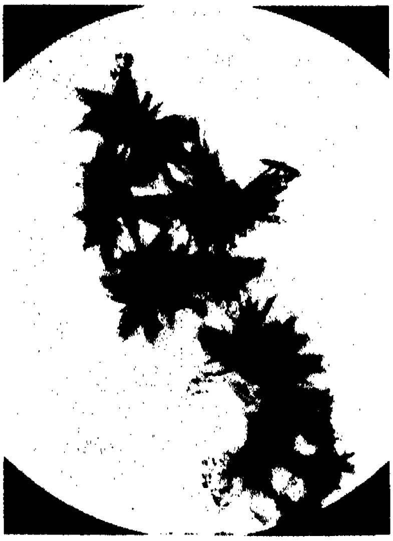

Figure 3

Figure 2. γ-Carotene from *A. javanicus* (Kniep's strain), crystallized from warmed methanol solution with a trace of water.

Figure 3. γ-Carotene from *A. javanicus* (Fiji D 2), crystallized from benzene solution by the addition of methanol.

Both approximately × 525 as here reproduced.

sporangia. Spores from resistant sporangia go through a distinctive process of encystment and liberate secondary swarmers which give rise directly to plants like their immediate parent. There is no obvious sexual generation, and anisogametes are not produced. A much reduced or microsexual stage may occur while the resistant sporangia are germinating, but sexual fusions have not been seen.

On the basis of these differences in life cycle, Emerson has divided *Allomyces* into two subgenera, *Euallomyces*, to include species with a marked morphological alternation of generations, and *Cystogenes*, to include forms such as *Allomyces moniliformis* which are characterized by cyst formation and the lack of an obvious sexual stage.

Our studies have shown that, under certain cultural conditions (notably on oatmeal agar—see Methods), *A. moniliformis* produces large quantities of orange pigment, often sufficient to mask the usual brown colour imparted by the resistant sporangia and to give the culture the appearance of a gametophyte of *Euallomyces*. The pigment is sometimes concentrated in large oil-drops, but it is not confined, as in *Euallomyces*, to any particular structures or parts of the plant: it occurs here and there, indiscriminately, within the cytoplasm of resistant sporangia, thin-walled sporangia, hyphae and spores. Orange pigment is not, so far as we have observed, ever produced by the closely related species *Allomyces cystogena*, although this form has a life cycle identical with that of *A. moniliformis*.

METHODS

All species of *Allomyces* can readily be grown in pure culture on a variety of substrata. On nutrient agar the fungus spreads vegetatively and forms large numbers of reproductive organs near the surface of the medium. If the cultures are placed in a fairly dry atmosphere, at an even temperature, to avoid condensation of moisture on the agar surface, the liberation of spores or gametes can be prevented, and pure asexual or sexual mycelia can be kept unmixed. The medium, found to be most satisfactory for growing both stages of *Euallomyces* for purposes of pigment extraction, was composed of agar (20 g.), soluble starch (15 g.), powdered yeast extract—Difco—(4 g.), K_2HPO_4 (1 g.), and $MgSO_4$ (0·5 g.) in 1 l. of water. Species of *Cystogenes* form richer growths and produce more reproductive organs on a medium made up with 50 g. of oatmeal per litre of water, steamed for 1 hr., strained, and solidified with 20 g. of agar. Hence this, rather than the yeast-starch medium, was used to prepare cultures of *Allomyces moniliformis* and *A. cystogena* for extraction.

In each experiment mycelium and reproductive organs of the fungus were scraped from the agar surface of a few (one to three) Petri dish cultures, placed in acetone, and stored therein, under an atmosphere of nitrogen. The flasks were agitated occasionally or allowed to stand, sometimes overnight, until all acetone-soluble pigment had been extracted. The completeness of the extraction was tested by additional washings with acetone, which usually removed only faint traces of adhering pigment solution, leaving the residual fungal matter colourless.

All pigment was then transferred from acetone to petroleum ether (boiling range 50–60° C) by diluting the acetone with water and extracting repeatedly with the petroleum ether.

The resulting petroleum ether solutions were subjected to the partition test by shaking, first with 90 % methanol, in which most free xanthophylls are preferentially soluble, and then with 95–96 % methanol, in which monohydroxy xanthophylls are preferentially soluble, i.e. hypophasic against petroleum ether. (The monohydroxy xanthophylls (1) cryptoxanthin, (2) rubixanthin and (3) gazaniaxanthin show absorption spectra virtually identical with (1) β-carotene and (2 and 3) γ-carotene respectively, but they appear in the hypophase of 95–96 % methanol when they are present (Kuhn and Grundmann 1934; Schön 1938). Then, after protracted treatment with hot solutions of potassium hydroxide in methanol, to saponify any xanthophyllic esters, the pigment was restored to petroleum ether and again given the partition treatments with 90 and 96 % methanol.

As a result of such treatment, all the hydrocarbon materials (carotenes) were retained in the epiphase of petroleum ether, while free xanthophylls, had they been present, would have been removed by the first alcoholic extractions, and esterified xanthophylls by the washings with the same solvents, following the hydrolytic operation.

The petroleum ether solutions were rendered free of methanol by a few successive washings with distilled water. When clear of all traces of suspended water droplets, the pigment was chromatographed by passing the solutions through Tswett columns of calcium carbonate (above) and calcium hydroxide or magnesium oxide (below). Calcium carbonate adsorbs the commoner xanthophyllic carotenoids, allowing carotenes to pass readily, while calcium hydroxide and magnesium oxide exercise differential adsorptive affinities for the various carotenes, giving rise to coloured zones on the column, corresponding to the positions of retention of the different isomers (Zechmeister and v. Cholnoky 1938).

In order to minimize the possibility of losses or alterations of the pigment by oxidation, the final stages of chromatography, preceding and during which the solvent was being drawn out of the column to final dryness, were always carried out in a current of coal gas. Saponification operations were effected under an atmosphere of nitrogen.

Careful mechanical separation of the coloured zones from such columns, followed by complete elution of the adsorbed pigment with petroleum ether containing a trace of pure methanol, resulted in a considerable degree of purification, and rendered the pigment ready for crystallization and spectroscopic examination.

For the spectroscopic work use was made of a Hartridge Reversion Spectroscope (manufactured by R. and J. Beck, London) of recent design, supplied with a symmetrical slit.

Careful calibration of the instrument by examination of the emission lines of mercury, sodium and lithium, and the absorption bands of a considerable number of known compounds (potassium permanganate, didymium nitrate, chlorophyll, cytochrome *C* and various other haematin derivatives, and a number of pure carotenoids), showed that it was possible to secure readings of a satisfactory order of accuracy for the identification of our pigments. The maximum deviation of our readings from true values lay within 1·5 mμ, or at most 1·7 mμ, while most of the determinations were of greater accuracy. Many of the same standard substances were examined with the same instrument independently by others (Mr S. W. Cole, Mr R. A. Beatty), who reported results of the same order of close agreement with corresponding true values.

It was frequently possible to secure improvement in the definition of absorption bands, and to increase the visibility of those bands in the violet region by interposing in the light beam a small blue-green Jena glass filter (BG 7; Schmelz-Nr F. 21426, from Schott and Gen., Jena) which transmitted most of the light in the blue-green to violet end and screened out most of it in the region of the longer wave-lengths.

The transmission values of this filter (for 1 mm. thickness), in the region which is of importance in our investigation, are given by the manufacturers as follows:

Wave-lengths mμ	Light transmitted %
775	1
700	1
644	5
578	24
546	50
509	75
480	81
436	72
405	59

Wave-length values for absorption maxima of pigment in carbon disulphide, as reported below, are averages of repeated readings, made both with and without the Jena glass filter in most instances, and often with varying slit widths. The averages of repeated readings taken under varying conditions of slit width, light filtration, and pigment concentration showed consistently close agreement with each other and with the true values for γ-carotene given as:

I, 533·5 mμ; II, 496 mμ; III, 463 mμ in carbon disulphide (Kuhn and Brockmann 1933*a*, *b*).

RESULTS

In order to gain a preliminary understanding of the pigments in *Allomyces* we have made extractions and analyses of ten different strains or isolates representing all the species now known, as well as three hybrids.*

The following observations and tests were made on the first four strains named below: (*a*) colour of growth on agar (based upon Ridgway's *Color Standards and Nomenclature*, Washington, D.C., 1912); (*b*) repeated extraction with hot and with cold acetone, and examination of solvent-free films of extracted lipoid material for traces of pigment; (*c*) treatment of chloroform solutions of the extracted lipoids with $SbCl_3$, also with concentrated H_2SO_4 (both reagents yield blue colours with carotenoids); the Liebermann-Burchard reagent (concentrated H_2SO_4 and acetic anhydride), which gives a series of colours ending in green in the presence of sterols, was employed for the demonstration of such compounds.

The forms which we have studied separately are given below, followed by the results of tests; for purposes of future reference, each individual strain is indicated according to its geographical source.

The asexual hybrids (no. 5 in the series) were given the tests described thereunder to determine something of the character of the brown pigment.

I. Euallomyces, *asexual generation*

1. *A. arbuscula* (Fiji F 1).
2. *A. javanicus* (Kniep's strain from Java).
3. *A. javanicus* (Fiji D 2).
4. *A. macrogyna* (Burma 3).

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* Two of the species mentioned here, *A. macrogyna* and *A. cystogyna*, have not hitherto been defined but will be described in a taxonomic treatment of *Allomyces* which R. E. is now completing. It is sufficient here to note that *A. macrogyna* is closely similar to *A. javanicus*, while *A. cystogyna* agrees essentially with *A. moniliformis*.

Colour: Russet to Mars brown.

Extraction: Solvent-free lipoid films showed only faintest traces of yellow colour.

Chemical tests: $SbCl_3$ negative; conc. H_2SO_4 negative; Liebermann-Burchard test positive.

Conclusions: Sterols always present, but no carotenoids in any of the above cultures.

5. *Hybrids* (S 31, S 34 and S 35): *A. arbuscula* (Burma 1 A) × *A. macrogyna* (Burma 3).

Colour: As above.

Extraction: No pigmented matter soluble in cold or hot water, cold or hot 0·1 N NaOH, dilute HCl, glacial acetic acid, acetone, absolute alcohol, or pyridine; much dark pigment extracted by hot, concentrated KOH, giving a yellow to brown, coffee-coloured solution; acidification of the filtered, clear, alkaline extract rendered it turbid.

Chemical test: An acidic, aqueous solution of $FeCl_3$, which gave a positive reaction (blue colour) with resorcinol in water, gave no reaction with the above acidified alkaline extracts of the fungus material.

Conclusions: No carotenoids were demonstrable. Notwithstanding the failure to detect the presence of phenolic bodies, the other properties of the extracted, brown substance showed that it belongs to the melanin series of pigments.

II. Euallomyces, *sexual generation*

1. *A. arbuscula* (Weston's stain frrom the Philippine Islands).

Colour: Less striking than other strains examined. Light ochraceous salmon or Capucine buff.

Extraction: Pigment removed only slowly by acetone, pyridine or ethanol. Fungal residue still contained traces of yellow pigment soluble in water and giving mild positive test for flavines by hydrosulphite bleaching followed by atmospheric reoxidation.*

Partition: Epiphasic to 90 or 95 % methanol, both before and after hydrolytic treatment.

Absorption spectrum in CS_2: Solution red-orange.

I, 532·5 mμ; II, 493·5 mμ; III, 464·5 mμ.

Conclusions: No true melanins; traces of flavines; small amounts of γ-carotene, but no xanthophylls or esters of these.

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* This test was kindly carried out by Dr D. E. Green.

2. *A. javanicus* (Kniep's strain from Java).

Colour: Capucine yellow.

Extraction: Acetone readily extracted all pigment, giving a rich yellow to orange solution.

Partition: All pigment epiphasic to 90 or 95 % methanol, before and after hydrolytic treatment.

Chromatograph: (1) A very pale, diffuse, pink zone remained in the $CaCO_3$ layer; (2) an orange zone migrated slowly through the $CaCO_3$ layer and became firmly adsorbed in the MgO column; (3) a trace of pigment migrated as a very pale yellow band through the MgO layer into the filtrate. (1) and (3) were in too small traces for investigation.

Chemical tests: Evaporation of the warmed eluting solvent (petroleum ether containing a trace of methanol) in a stream of nitrogen gave many microcrystals possessing the characteristic odour of carotenes. Chloroform solutions gave the following confirmatory tests for carotenoids: (1) blue colour with $SbCl_3$, (2) blue colour with concentrated H_2SO_4.

Crystallization: Dissolved in hot methanol, the pigment, on cooling, and with the addition of a trace of water, gave dark red microcrystals which were formed in bundles of radiating spindles (see plate 16, figure 2).

Absorption spectrum in CS_2: Three absorption bands observed:

Unchromatographed material:

I, 530 mμ; II, 494 mμ; III, 465 mμ.

Chief eluted fraction of chromatographed pigment:

I, 534 mμ; II, 495·5 mμ; III, 464 mμ.

Conclusions: No melanin pigment; no xanthophylls or xanthophyll esters but only γ-carotene, accompanied by a faint trace of another carotene.

3. *A. javanicus* (Fiji D2).

Colour: Orange. (A large majority of the gametangia in this strain are male, hence the colour is particularly intense.)

Extraction: All pigment readily extracted with acetone; no indications of melanin in the remaining fungal material.

Partition: Epiphasic to both 90 and 96 % methanol, before and after hydrolytic treatment.

Chemical tests: Chloroform solutions yielded a blue colour upon treatment with either $SbCl_3$ or concentrated H_2SO_4.

Chromatograph: Faint traces of pink in $CaCO_3$ layer; chief carotene fraction settled at top of MgO layer in red-orange band, while a paler zone of similar colour gradually developed a little way below. Filtrate colourless.

Crystallization: The main carotenoid constituent, which was γ-carotene (vide infra), was crystallized out of a small amount of benzene by addition of methanol (cf. Winterstein 1933) as minute bundles of needle-like spindles (see plate 16, figure 3).

Absorption spectra in CS_2: (*a*) Upper copious zone from MgO part of chromatogram: red-orange in CS_2; three-banded spectrum:

γ-carotene: I, 532 mμ; II, 494 mμ; III, 462 mμ.

(*b*) Lower, paler zone from MgO chromatogram: orange in CS_2; two-banded spectrum with bands less sharply defined:

β-carotene: I, 518–519 mμ; II, 483 mμ.

Remarks: Using a new set of cultures of this strain we repeated the chemical investigation with the exception of the hydrolytic treatment. Spectroscopic examination of the γ-carotene gave identical maxima as previously, while the small amount of β-carotene gave essentially the same maxima, i.e. I, 520·5 mμ; II, 485 mμ.

Conclusions: No melanin. The carotenoid pigment, as in previous cases, contained no xanthophylls or xanthophyll esters, but was composed of considerable quantities of γ-carotene and a small amount of β-carotene.

4. *A. macrogyna* (Burma 3).

Colour: Zinc orange.

Extraction: Readily with acetone.

Partition: Entirely epiphasic to methanol solutions, both before and after hydrolytic treatment.

Chromatograph: Orange band travelled slowly through $CaCO_3$ layer and settled at top of MgO; faint traces of coloured material, remaining in $CaCO_3$ layer, and a very pale, diffuse zone, which travelled below the main discrete orange band into the MgO, were too small in quantity for further investigation.

Crystallization: Readily brought about by cooling methanol solutions of the pigment and adding a trace of water. Crystals of same habit as those described and photographed from *A. javanicus* (as shown in figure 2).

Absorption spectrum in CS_2: Usual three bands:

I, 533·6 mμ; II, 495 mμ; III, 463 mμ.

Conclusions: No melanins present; much γ-carotene, and a faint trace of another carotene; no xanthophylls.

5. *Hybrid* (S 31 *b*): *A. arbuscula* (Burma 1 A) ♂ × *A. macrogyna* (Burma 3) ♀.

Colour: Orange. Pigment not diffusible into agar substrate. Direct preliminary examination of culture with spectroscope showed a broad diffuse band in the blue-green, with centre at about 522 mμ.

Extraction: Direct treatment with petroleum ether extracted no pigment. Acetone readily extracted all pigment, giving a rich yellow solution.

Partition: Both before and after treatment with hot, methanolic KOH solution, the pigment was epiphasic to 90, 95, and even 99 % methanol.

Chromatograph: In $CaCO_3$ layer above, no bands, but only a very faint, pinkish, diffuse zone, bearing an insufficient quantity of pigment for investigation.

In $Ca(OH)_2$ layer beneath: (1) a pale pink zone at top of layer, yielding very little pigment on elution; (2) a single, broad, discrete, orange zone a little way below the upper one; column beneath second zone, as well as filtrate, colourless, even after development of chromatograph with benzene-petroleum ether mixtures.

Absorption spectrum in CS_2: Whole pigment, directly following hydrolysis, but before chromatographing, showed the same spectrum as did the chief fraction from the chromatogram, viz: I, 533 mμ; II, 495 mμ; III, 463 mμ. Eluted pigment from (1), upper, pale zone of $Ca(OH)_2$ column, gave a red-orange solution in CS_2; its bands were not sharp: I, *ca.* 535 mμ (barely perceptible); II, 474·5 mμ (diffuse). Eluted pigment from (2), single, lower, rich orange zone, gave a spectrum of three bands: I, 533 mμ; II, 495 mμ; III, 463 mμ.

Remarks and conclusions: γ-carotene was the only pigment identified, and was present in considerable quantity; no xanthophylls or their esters were present. Melanin pigments were absent.

III. Cystogenes (*no obvious alternation of generations*)

1. *A. cystogena* (Burma 1 B).

Colour: Ochraceous tawny.

Careful, repeated extraction of this material, followed by examination of the lipoid matter and the application to it of the chemical tests used in studies of the other strains failed to reveal the presence of any carotenoid compounds, although sterols were demonstrated.

2. *A. moniliformis* (Wolf's strain, Mexico 46).

Colour: Ochraceous salmon.

Extraction: Some pigment was yielded to acetone, imparting to the latter a pale yellow colour.

Partition: Pigment entirely epiphasic in petroleum ether when shaken with 90 or 96 % methyl alcohol, both before and after hydrolytic treatment.

Chromatograph: All pigment passed through $CaCO_3$ layer, becoming adsorbed at the top of the MgO layer as a pink-orange zone.

Absorption spectrum in CS_2: Orange-yellow solution; three bands:

I, 536 mμ; II, 497 mμ; III, 464 mμ.

Conclusions: The only carotenoid pigment detected in this form was γ-carotene.

DISCUSSION

The following general conclusions can be drawn from the results of extractions and tests described above. (1) The brown colour of the resistant sporangia of *Allomyces* is almost certainly due to the melanin class of pigments. This conclusion is based upon the chemical tests applied to three cultures of asexual material (Hybrids S 31, S 34 and S 35) and upon the close similarity in the structure and appearance of resistant sporangia of all species (*Cystogenes* as well as *Euallomyces*). (2) Asexual plants of *Euallomyces*, grown on the medium which we used, were essentially lacking in carotenoid pigment; no traces of carotenoids were detected in extracts of asexual material, although complete extraction was consistently indicated by the demonstration of sterols. (3) Sexual plants of *Euallomyces* formed quantities of a carotene, which, by ordinary inspection, varied in amount according to species but was identical in chemical behaviour and absorption spectrum with γ-carotene. (4) Of the cyst-forming *Allomyces*, which lack an obvious sexual stage, *A. cystogena* contained no detectable traces of carotenoid pigment, while *A. moniliformis* yielded γ-carotene but no other carotenoids.

In all cases the carotene extracted passed readily through calcium carbonate, and by far the major part of it appeared as a single, discrete band, always at or near the top of the calcium hydroxide or magnesium oxide component of chromatographic columns. A second carotene, less easily adsorbed and therefore passing the chief zone on the column, was occasionally observed, usually in very small traces; in one strain of *A. javanicus* (Fiji D 2) a secondary carotene of this sort was present in sufficient amount to identify it spectroscopically as the β-isomer. Traces of other coloured zones sometimes appeared as well, but these pigments were usually in too small quantities for further study. It is significant that the total carotenoid gave the same sharp spectrum, with bands in the same respective positions, before chromatography as did the greatly preponderating fraction thereafter.

The chief pigment, crystallized in clusters of dark red, needle-shaped microspindles, as described above (see Results), underwent rather ready decomposition on covered glass slides, and, within an hour or two, showed incipient lack of crystalline definition, with gradual formation of minute, adhering, yellow or colourless droplets of oil. This degradation could be followed under the microscope, and the oil-droplets which formed are clearly visible in figure 2. Even when stored in methanol solution, under nitrogen and in a dark refrigerator, the crystals decomposed gradually, progressing within a week or ten days to an amorphous condition with bleaching of colour.

At no time were any free or esterified xanthophylls detected;* the pigment extracted remained almost completely epiphasic, even against methanol solutions whose concentrations were 98 % or higher and in which the petroleum ether itself was partially soluble.

γ-Carotene has an absorption spectrum virtually identical with that of its two known monohydroxy derivatives, namely, rubixanthin (Kuhn and Grundmann 1934) and gazaniaxanthin (Schön 1938). In the partition test, however, both rubixanthin and gazaniaxanthin, in contrast with γ-carotene, behave hypophasically when 95 % methanol, or greater concentrations, are employed against petroleum ether solutions (Zechmeister and v. Cholnoky 1938; Schön 1938). Schön found that his crystals of gazaniaxanthin appeared from benzene-methanol mixtures in the form of deep red, rectangular leaflets, whereas those of rubixanthin appear, without methanol, as lighter, reddish brown prisms.

The epiphasic behaviour of our pigment, both before and after hydrolytic treatment, against solutions of 95–99 % methanol, distinguishes it clearly from rubixanthin or gazaniaxanthin; furthermore, its crystalline habit differs considerably from the former and radically from the latter. Its partitional and chromatographic behaviour show that it is a carotene, and its spectrum is identical with that of only one carotene known at present, namely, the γ-isomer.

Like α- and β-carotenes, the γ-compound possesses vitamin A potency. It is rare in plants, constituting only a very small fraction of most total carotene preparations from ordinary sources (Kuhn and Brockmann 1933*b*). Besides our male *Allomyces* material, a few other relatively rich plant sources of this isomer include the skins of the tropical fruit of *Gonocaryum pyriforme* (Winterstein 1933), the marsh dodder *Cuscuta salina* (MacKinney 1935), and certain fungi and bacteria (Lederer 1938, and others cited by him).

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* The absence of xanthophyllic carotenoids in *Allomyces* is in agreement with the observations of Lederer (1938), who reports his failure to detect xanthophylls or their esters in any of the species of fungi which he examined. In exceptional cases, he found various carotenes accompanied by one or another of the rather rare monohydroxy compounds such as rubixanthin.

It will be of value to mention briefly here the few investigations which bear upon the occurrence of carotenoids in relation to reproductive structures and the processes of reproduction in plants. More than forty-five years ago Zopf (1892) observed that the yellow pigments, stored in large amounts in the spores of many Rusts and certain Mucors, disappeared and, as he believed, were utilized during germination. He suggested, therefore, that they might be acting as reserve food-substances, and likened them to the carotene of the carrot, to which he ascribed the same function. Kohl (1902) accepted this idea; but, as Palmer (1922) says, "it is perhaps safer to admit merely that they (the carotenoids) often occur in association with reserves".

Emphasis has been placed upon the sexual and reproductive aspects of carotenoids by more recent investigations. Lendner (1918) believed that more yellow pigment was always stored in the large (+) gametes of the heterothallic mould *Mucor hiemalis* than in the smaller (−) gametes. He suggested that the more vigorous and highly pigmented (+) strain might well be considered to be the female. Satina and Blakeslee (1926*a*), after comparing the (+) and (−) strains of a wide variety of Mucoraceae, concluded that (+) races showed, fairly consistently, a higher lipoid content and more yellow pigment than did the (−) races. They, like Lendner, believed that the (+) and (−) strains of Mucors correspond, respectively, to the female and male of other plants, in which the sexes are more highly differentiated. This relationship between pigmentation and sexuality in the Mucoraceae was further stressed by Chodat and Schopfer (1927), when they reported the results of their investigation of carotene in *Mucor hiemalis*. They expressed the view that the higher carotenoid content in (+) races of Mucors reflects a general condition of more intense metabolism and greater storage of food reserves in female than in male plants.

Satina and Blakeslee (1926*b*), while investigating numerous differences between the sexes of a variety of dioecious phanerogams, found that the leaves of females yielded more yellow pigment to 60% alcohol than did those of male plants. Some years later Murneek (1934) showed that the leaves of spermatophytes such as *Cosmos*, *Salvia*, and *Soja* contained about the same amounts of chlorophyll whether they had been exposed to long or short photoperiods, but short-day plants flowered sooner and had more carotenoid pigment in their leaves than did long-day plants. Both long- and short-day plants, while changing from the vegetative to the reproductive state, showed a progressive increase in the amount of carotene and xanthophyll; the carotenoid content of their leaves reached a maximum at the time of flowering and then decreased.

The orange colour of the male gametes in *Allomyces* is comparable to similar pigmentation occurring in the male cells of many algae and some other cryptogams. During the course of our investigation, we learned (personal communication to D. L. F.) of the interesting study of pigments in the sexual cells of two brown algae, *Fucus serratus* and *Ascophyllum nodosum*, being carried on by Professor I. M. Heilbron and Dr E. R. H. Jones at the Imperial College of Science and Technology. These investigators have found that although the female gametes contain considerable amounts of chlorophyll and fucoxanthin, they are without carotenes, while the male gametes, on the contrary, are pigmented with carotene(s) exclusively.

Another parallel example of pigmentation, of special interest from the phylogenetic stand-point, is that which occurs in the primitive fungus *Blastocladiella* (*Rhopalomyces*) *variabilis*. In this water-mould, which is clearly very closely related to *Allomyces*, Harder and Sörgel (1938) have discovered sexual reproduction and a type of alternation of generations very similar to that of *Euallomyces*. In contrast with *Allomyces*, however, *Blastocladiella* bears isogametes on dioecious sexual plants; hence Harder and Sörgel have designated the sexes, or sexual strains, as (+) and (−) rather than female and male. The point which is of interest here is that the plants of one strain produce orange-coloured gametangia and gametes, while the sexual cells of the other strain are unpigmented. The pigment has not yet been identified chemically. Perhaps those who have worked with the (+) and (−) strains of the Mucors might be inclined to term the orange gametes of *Blastocladiella* female, but it seems much more logical to liken them to the pigmented male gametes of *Allomyces*.

Microscopic observations of the orange pigment in *Allomyces* indicate that, whatever part it may play, it is not required in more than small amounts and is, therefore, very probably not serving merely as a reserve food-substance. In pure water cultures of young sexual plants, gametes are discharged as rapidly as they are formed, and sometimes only the faintest traces of orange colour appear in the male cells; yet normal sexual fusions take place, and the zygotes germinate readily under these natural conditions, notwithstanding the small amount of pigment developed. In older water-cultures or on agar media, where gamete discharge is prevented by the high concentration of organic matter or the lack of moisture, the carotene accumulates in quantities in excess of those which ordinarily occur in nature.

The studies of Kuhn, Moewus and Jerchel (1938) and Moewus (1939) have recently brought the question of carotenoids in relation to sexual reproduction into great prominence. These workers have indicated the specific part played by crocin in promoting the fusion, in light, of gametes of the green alga *Chlamydomonas eugametos*.

Our investigations have not demonstrated a metabolic role played by γ-carotene in sexual reproduction, but the absence of carotenoids from the asexual stage of *Allomyces*, the regular occurrence of carotene in the sexual stage, and its restriction to the male reproductive cells (excepting the unique condition in *A. moniliformis*) further emphasize the possibility that carotenoid pigments play an important part in reproductive processes.

We gladly take this opportunity to express our thanks to Professor Sir F. G. Hopkins, of the School of Biochemistry, and Professor F. T. Brooks of the School of Botany, for their continued interest and encouragement during this work. We are also indebted to various other members of each School for their numerous courtesies and their willing assistance in many ways.

SUMMARY

In the life cycle of certain species of the aquatic phycomycete *Allomyces* an asexual or sporophytic generation regularly alternates with a sexual or gametophytic one. Asexual plants bear thin-walled, colourless zoosporangia and thick-walled, brown resistant sporangia; sexual plants bear colourless female gametangia and orange male gametangia.

The pigment in the resistant sporangia is confined to the heavy, pitted, outer wall; the inner membrane, the cytoplasm, and the spores which are formed are all colourless. The orange pigment, found exclusively in the male cells of the sexual phase, is present in oil-droplets within the cytoplasm and is still apparent in the male gametes after their emergence from the gametangia. There is not any trace of pigment in the female gametes, which, although more than twice the size of the males, are similar to the latter in structure and motility.

Results of the present chemical investigation of the pigments in *Allomyces* may be summarized as follows. The brown pigment of the resistant sporangia belongs to the melanin group, and careful examination

and tests have failed to reveal any detectable traces of carotenoids in the solvent-free material extracted from sporophytic plants. The brilliant, orange colour of the male cells in the sexual generation, on the other hand, is clearly due almost entirely, in the majority of cases examined, to the presence of carotenoid pigments. Melanins are rare or lacking in this sexual phase. No traces of oxygen-containing carotenoids, i.e. xanthophylls, xanthophyll esters, or carotenoid acids have been detected. γ-Carotene was found in remarkably high concentrations, accompanied in certain strains by small amounts of other isomers such as β-carotene. The pronounced and nearly exclusive synthesis of the relatively rare γ-isomer of carotene is of particular interest.

These findings concerning the pigmentation of *Allomyces* are in close accord with existing information: the synthesis and selective storage of carotenoids or their derivatives in structures associated with reproduction is well known in many other cryptogams as well as in countless higher plants. Notable also is the corresponding concentration of carotenoid pigments in the reproductive structures and secretions of many animals. The foregoing study lends emphasis to the probability that such compounds play important biochemical roles in sexuality and the processes involved in the metabolism of reproduction.

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The upper frequency limit for the binaural localization of a pure tone by phase difference

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Introduction

It is well known that if a pure tone is presented binaurally so that its intensity is the same in each ear, and there is no phase difference between the notes at the ears, the resulting sensation is that of a source of sound located in the median plane and situated either within the skull or outside it. If a difference of phase between the notes is gradually introduced without altering their intensities, and the frequency of the tone is, say, 800 cyc./sec., then the source appears to move laterally towards the side of the ear in which the phase is made to lead. According to some observers, the movement is on a horizontal circular arc centred between the ears, while others consider it to be along a line joining the ears. There is general agreement among workers on localization that considerable difficulty exists in the detection of such movements at frequencies above 800 cyc./sec., and that their extent is more limited, but they disagree as to the frequency above which the lateral motion ceases to be noted. Stewart (1920) obtained movement at 1280 cyc./sec., but not at 1536 cyc./sec.; Banister (1925) found movements at 1040 and 1345 cyc./sec., but anomalous in character. On the other hand, von Hornbostel and Wertheimer (1920) state that at about 800 cyc./sec., the lateral movement no longer extends 90° left and right, but decreases as the frequency increases, and should reach zero at 17,000 cyc./sec. Halverson (1927) found that observable right and left effects appear to be present up to the upper auditory limit. Below 1400 cyc./sec. judgments of direction are fairly consistent, and between 1400 and 3000 cyc./sec. there is no particular difficulty if allowance is made for the more rapid onset of fatigue at these frequencies. Above 3000 cyc./sec. lateral effects are still observable, though median localization is extremely difficult to achieve. Hartley (1919) gave theoretical curves showing the phase difference produced at the ears by sources of various frequencies at different distances from the head. According to these, above 650 cyc./sec. the maximum movement of the apparent source would be less than 90°, though at 1860 cyc./sec. there should still be about 27° of movement on either side of the median plane. These curves have been checked experimentally by Firestone (1930), who found for three frequencies the phase difference and amplitude ratio of the sounds entering the ears of a man-shaped wax dummy from a source at varying azimuths around the head and at different distances from it. The phase differences found were in good agreement with the values calculated by Hartley.

Various methods have been used to present the notes to the ears and to vary the phase difference between them. In the experiments of Halverson the sounds were produced by a tuning fork and led to the ears through tubes, in one of which was incorporated a sliding section to vary its length and thus the phase difference at the ears. An objection to this is the possibility of resonances in the tubes, different on both sides on account of their different lengths, so that intensity changes might occur as well as changes of phase. Stewart, and Banister, used notes generated electrically and presented to the ears by telephones. Continuous phase changes could be made with their generators, but the purity of the resulting notes was doubtful.

In the present experiments electrically generated pure tones were used, and the changes of phase were brought about by an electrical method free from the objections to those previously mentioned. To investigate the upper limit for localization by phase difference the least change of phase which would produce a detectable lateral movement of the apparent source from the median plane was determined for each observer at frequencies from 600 to 2000 cyc./sec., so that the accuracy with which such changes could be detected and any variation with frequency in the amount of change required could be found.

It is shown that at medium frequencies, up to about 1000 cyc./sec., definite shifts in apparent position of a source of sound can be produced by phase differences alone when equality of intensity at the two ears is rigidly controlled. Above this frequency this localization by phase difference (or more exactly time difference) becomes more uncertain, and above about 1400 cyc./sec. it entirely fails. The basis of normal auditory localization for medium frequencies is thus the time interval between the arrival at the two ears of corresponding phases in each cycle of the sound wave.

This view has become increasingly probable in the light of recent researches, such as those quoted above. In particular Shaxby and Gage (1932) show that loudness differences at the two ears can play no significant part in localization near the median plane. Yet the fact that differences of loudness at the two ears may under certain conditions affect localization (e.g. H. E. O. James 1936; J. W. Hughes 1939) has left some measure of doubt as to whether a source of sound can be localized by phase difference alone in the absence of any intensity difference. The present paper shows conclusively that it can.

APPARATUS

The pure tone was generated by a heterodyne oscillator, the output stage of which supplied a quadrature circuit which gave, after adjustment at the particular frequency used, two e.m.f.'s of equal magnitude differing in phase by 90° (Gage 1934). These were used to energize a pair of phase-shifting transformers (Gage 1934), and from the secondary coil of either of these it was possible to take an e.m.f. the phase of which could be continuously varied by more than 360° without any significant change in its magnitude. The e.m.f.'s available from the two coils were applied to separate amplifiers in each of which were incorporated two intensity controls, one to increase the output in steps of 5 db. and the other to give a continuous variation of the output between these steps. The output from each amplifier actuated one of a pair of telephones worn by the observer in the "sound-proof" room in which the experiments were carried out. The cathode-ray oscillograph method of adjusting the quadrature circuit used by Gage was replaced by one in which the reading of a valve voltmeter across the output of one of the amplifiers was arranged to be constant for all positions of the secondary coil in the corresponding phase-shifting transformer. This method, in addition to the greater ease and speed of adjustment which it provided, also enabled it to be made more accurately, since the intensity variations for a complete rotation of the coil were reduced from ± 0·8 to ± 0·2 db.

PROCEDURE

In the first experiments, carried out with three observers familiar with the phenomenon involved, the procedure was as follows. The monaural threshold settings of the intensity controls on each amplifier were found by a method previously described (Hughes 1938). Then the output of each amplifier was raised 40 db. by the main intensity control, and the two outputs adjusted to be in phase by a method described later. Provided that the two notes were equally loud, the observer would then have the sensation of a source in the median plane. In practice some slight difference in loudness was usually present, due to inequalities in the intensity controls and/or inaccuracies in the threshold settings, and the fine controls were used to make the source appear "central". This process was facilitated by an intercommunication system by which the observer could speak to the assistant operating the controls outside the room, or vice versa. A mercury switch enabled the observer to short-circuit the telephones and obtain silence if necessary; "centralization" was often easier if the note were heard only for short intervals during the process. When this had been carried out to the observer's satisfaction, he signalled that he was ready; the assistant signalled back and almost at once started to change gradually the phase of the note from one phone by rotating the dial of its phase-shifting transformer. When the observer detected a movement of the source from the median plane he signalled again; the dial reading was noted, the phases of the two notes rapidly equalized so that the "swing-back" of the source was made apparent, and another change in the opposite direction was made in a similar way. Ten changes were made altogether at any one frequency. The rate of alteration of phase was usually about 10° per second, but it was varied so that the observer was discouraged from subconscious "timing" of his signals. After a rest, the frequency was changed, the quadrature circuit readjusted, and the whole procedure repeated.

For the second group of observers, after tests with the first group had shown that there was no significant variation of the changes detectable at any particular frequency with the intensity of the notes used, the determination of the thresholds was dispensed with, and the mean settings of the controls found for the first three observers were employed. This shortened considerably the time taken for a set of experiments.

There is some difficulty in devising a method suitable for the rapid adjustment of the telephone outputs to zero phase difference at various frequencies. In the experiments described, it was arranged that the voltages applied to the two output circuits were in phase; this was carried out once and for all at each frequency by applying the output voltages from the amplifiers each to one pair of deflecting plates of a cathode-ray oscillograph and adjusting one of the phase-shifting transformers until the elliptical trace usually produced became a straight line. This adjustment was independent of intensity, as was shown by the fact that considerable variation of the intensity controls failed to open the oscillograph trace into a narrow ellipse. Hence the setting of the transformer to the reading thus determined (with the other at, say, 0° each time) ensured that the output voltages were in phase at all settings of the controls used in practice. Since the output circuits consist of high resistances in series with the small inductances of the phones, the currents in them will then differ little in phase even if the inductances are not exactly equal. There may still be a phase-lag between their sound outputs even if the currents in the phones are cophasic, but the use of two similar phones reduces the error due to this, as well as tending to equalize the inductances, and it was assumed that the sound outputs were cophasic in the apparatus used when the output voltages had been adjusted in the way described.

After the majority of the experiments had been performed, this method of finding the transformer settings for cophasic sound outputs was checked by another, similar to that used by Wightman and Firestone (1930). The two telephones, fitting into sponge-rubber caps, were held by elastic bands against two opposite faces of a block of wood of about 2 in. cube through the centre of which a $\frac{1}{4}$ in. hole had been drilled; the openings in the earpieces were arranged to be opposite the ends of this hole. Into a similar hole meeting the first at right angles and symmetrically placed with respect to its ends was fitted a tube leading to a pair of stethoscope binaurals. By fixing the intensity of the note from one phone at the level used during the experiments and varying in turn the intensity and phase of the other it was possible to adjust the two notes with some accuracy to phase opposition. A rotation of one of the transformers through 180° then brought the notes into phase. This process could also be carried out once and for all at the frequencies used, and the settings of the phase-shifting transformer were much the same as those given by the previous method. The accuracy of the settings decreases with the intensity of the notes, but at 50 db. above threshold the phase-opposition position could be determined to within ± 1°.

It will be noted that the sound outputs are equalized in intensity by the latter method of setting, though their loudness will not be the same unless the thresholds in the two ears are equal. Because of this it was usual to make a further adjustment of the intensity for one ear to arrange that the note was "central" when the phase difference at the ears was zero. Some experiments made previously to those described here (Hughes 1939) had indicated that the apparent position of the source could be made to move laterally in a way similar to that caused by phase variation if the intensity at one ear were raised or lowered while the two notes remained in phase, and in several cases this "centralization" had to be carried out with some care if the ease of detection of apparent motion of the source with variation of phase was to be similar for both directions. It had also been found that "centralization" by change of intensity was more certain if the phase difference between the notes was not zero, but 180°, though the least perceptible gradual changes of intensity actually measured for both phase relations showed little significance from one another. For the three experienced observers the "centralization" was therefore carried out at 180° phase difference, while for the others the zero phase difference position was used.

RESULTS

The results of the experiments are indicated in the diagrams, in which the least perceptible gradual change of phase from zero phase difference has been plotted against the frequency at which the changes were determined. Each point on the diagrams represents the mean of ten experimentally determined phase changes, five in one direction and five in the other, taken alternately; the frequencies were not always employed in ascending or descending order, since sets of readings were often taken at frequencies 200 or 400 cyc./sec. apart, and the remainder taken later. In the first diagram the results for the three experienced observers are shown, while the second indicates those for five observers unfamiliar with the phenomenon involved. Though not shown in the diagrams, it may be noted that the standard deviation for a set of ten changes rose considerably as the mean phase change increased; for one observer (W. L. B.) while the standard deviation for a mean change of 15° was ± 4°, it had risen to ± 9° at 33°, and to ± 32° at 127°, and similar results were found for the others. For this reason no attempt has been made to join the points by a smooth curve.

On the theory that difference in time of arrival at the ears between the corresponding parts of the wave is the basis of localization, it would be expected that, in the absence of any other effect, the least perceptible difference of phase would increase with frequency, so as to give a constant least perceptible time difference at the ears. In the results of the experiments for the first group of observers there appears to be a decrease in the least perceptible difference of phase until the decrease is countered by the rapid increase due to inability to detect changes of phase at the frequencies concerned, but the deviation in the settings and the absence of data for frequencies below 600 cyc./sec. preclude any definite deduction from this. The observers of the second group show no decrease in this way.

The comments of the observers on the nature of the change at the higher frequencies are of interest. All remarked on the increasing difficulty of deciding whether a change had occurred as the frequency of the notes increased (this is reflected in the greater deviations among the settings), and considered that the nature of the change when it was detected was different from that which prompted them to signal at the lower frequencies. All except two of the observers reached a limiting frequency in the region of 1400 cyc./sec. above which no phase change whatever could be detected, no matter how large it might be. Changes could be detected at a frequency 100 cyc./sec. lower than the limiting frequency, but many were missed altogether, and the last point plotted for each observer represents the mean of about six or seven changes rather than of ten; the others, though as much as 360°, had not been noticed. Two observers (W. L. B. and J. H. S.) considered that the rotation of the phase-shifting transformer produced a change of some kind which persisted up to 2000 cyc./sec., though a test at 5000 cyc./sec. indicated that it was no longer present. There was a good deal of uncertainty as to the phase difference at which this change occurred, and it was often not noticed at all. Two observers (W. L. B. and D. C. G.) stated that at 1400 and 1200 cyc./sec. respectively the direction of apparent motion of the source was the same for both directions of rotation of the phase-shifting transformer. On further consideration one (W. L. B.) thought that the change consisted of a small movement in one direction and then a return to the central position with a large movement in the other.

The diagrams show that the mean changes required for the observers unfamiliar with the phenomenon are greater than those for the experienced observers. With two exceptions the former group required little fine adjustment of intensity for the preliminary "centralization", they nearly always accepted the approximately equal intensities presented to them without requiring any change. In the case of T. Sh., inconsistencies in the "Right" and "Left" changes were present until the centralization had been carefully carried out, and B. G. O. was quite sensitive to intensity variation. This observer, though without previous experience of the work, rapidly adapted himself to it, and gave quite definite indications as to the adjustments he required to produce "centrality".

FURTHER EXPERIMENTS

In order to obtain a check on the results found by the experiments described, some further work was carried out by the three observers constituting the first group. In the first instance an abrupt, rather than a gradual, change of phase was brought about by a rapid rotation of the transformer dial. After "centralizing" in the usual way at any particular frequency, the observer was given a signal, after which he was to state whether he had detected an abrupt change of location or not. Five such changes were interspersed with five signals which were not followed by a change; the order in which the changes were made was unknown to the observer, who was, however, aware that five of the signals were "controls". In every case the change was made within about 5 sec. of signalling, and the five changes in any one set of ten signals were of the same magnitude and in the same direction. After each reply on the part of the observer the original conditions were restored and a few seconds allowed to elapse before the next signal to allow the observer to recover the sensation of a source in the median plane. Sets of ten signals were given at each frequency for phase changes of different amounts until the least change which the observer was able to detect four times out of five, with a similar record for "no-change", was found. The results obtained at the various frequencies show a rise in the least change detectable as the frequency increases and at 1300 and 1200 cyc./sec. respectively J. W. H. and J. H. S. were unable to detect large phase shifts by this method. At about the same frequency W. L. B. found large changes necessary, though at all frequencies he was more uncertain in his judgements than were the other two. J. H. S. was able to detect that some kind of a change occurred at 4000 cyc./sec., but had no sense of location at this frequency, and the change may have been caused by an error in the adjustment of the quadrature circuit, since this becomes more difficult as the frequency increases.

Two observers (W. L. B. and J. W. H.) carried out a variation of the experiments described above. In this case the abrupt change of phase was kept constant at 90° and the accuracy with which it could be detected was tested at various frequencies. After "centralization" the observer switched off, signalled that he had done so, and the operator rapidly made the change in one direction or the other or left the phase unaltered. He then signalled back, and the observer switched on again and stated whether the source was now Right, Left, or Central. The interval between switching off and on again was always short (one or two seconds). Five changes in each direction were interspersed with ten "no-changes" in a way decided upon by the operator and unknown to the observer. Care has to be taken with the "centralization" in these experiments, but when this was properly carried out both observers were able to obtain 80 % correct results for each position in a set of twenty signals at the lower frequencies. However, as the pitch of the note was raised the difficulty of detection increased, and at about 1400 cyc./sec. both observers broke down and gave judgements which they themselves felt to be uncertain, and which were in fact quite inaccurate.

CONCLUSIONS

The results of the experiments appear to show that an upper limit for binaural localization by phase difference does exist, so that for frequencies higher than 1400 cyc./sec. observers fail to detect any change of localization when the relative phases of the two notes are varied while their intensities remain unchanged. This is true both for observers familiar with the phenomenon associated with change of phase and for those who are unaccustomed to it, and while the frequency at which localization becomes difficult varies slightly from one observer to another, the three methods employed give consistent results with any one observer.

In connection with experiments on similar lines, it has been suggested by Banister (1925) that the results found, which agreed very well with the expectations of the experimenters, who were also the observers, were due to subconscious suggestion on their part, and that observers unaware of the results to be expected would fail to repeat them. It was felt that in the present work, although in the preliminary experiments the observers were not familiar with the results of previous workers, similar objections might be raised, and for this reason the procedure followed throughout was evolved. In this way any tendency on the part of the observer to make his settings conform with his expectations could be checked by varying the rate at which the relative phases of the notes were changed. When the second group of observers was used, care was taken to give them no idea as to the nature of the investigation; they were familiarized with the effect to be noted without any indication that it might vary as the frequency of the notes was raised. Nevertheless, they commented spontaneously on the increased difficulty of detecting phase-change as the frequency increased, and though they received non-committal replies, they all showed extreme uncertainty in the region of 1400 cyc./sec. as the results indicate. Absence of the operation of any factor of suggestion with the experienced observers is confirmed by the results of the experiments with sudden phase change and detection of a fixed change at different frequencies, since in these it was impossible for the observer to attain the requisite accuracy unless he was signalling changes he actually heard.

The localization of a source of sound by phase (time) difference implies of course that a distinct event of stimulation occurs in the auditory nerve at some fixed period in each exciting sound wave.* The interval between these events for the right and left ears is interpreted by some cortical mechanism or process as localizing the position of the source of sound relative to the median plane of the body. If then the successive cycles of the sound wave follow each other at an interval of time equal to or less than the refractory period for the nerve this mode of localization must fail. The graphs of figures 1 and 2 indicate that localization by phase difference does reach such a limit at a frequency of about 1000 cyc./sec., i.e. for a time interval of 1σ. At a frequency of 1400 cyc./sec., or a time interval of 0·7σ, localization has become exceedingly poor or even impossible.

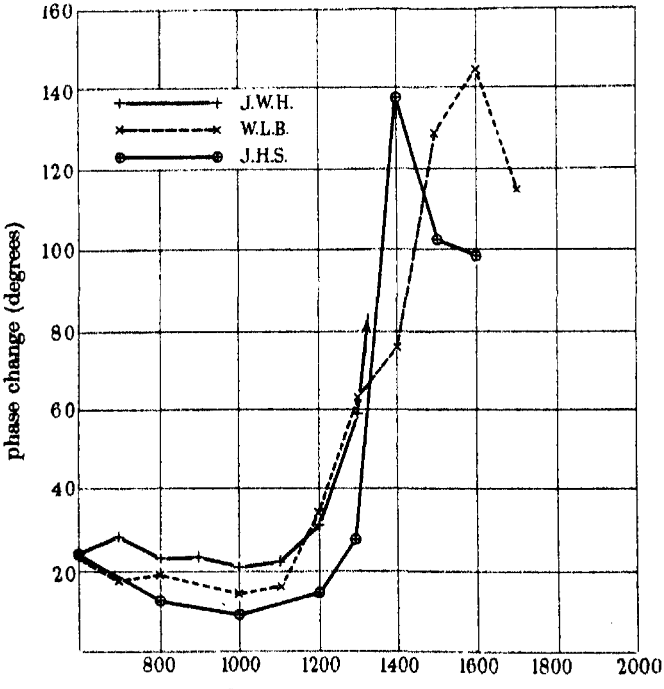

FIGURE 1

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* The phase concerned is probably that of production of negative pressure in the auditory canal (Hallowell Davis 1935).

Thus the VIIIth nerve seems to have much the same refractory period as other nerves, motor and sensory. For example, the absolute refractory period of the sciatic nerve of the frog is about 2σ at laboratory temperatures, falling to about 0·6σ at 40° C. At 5° C it is not less than 3·5σ (Bugnard and Hill 1935). Amberson (1930) obtained similar results, his mean values ranging from about 3σ at 10° C to 0·5σ at 27° C, with about 1σ at 20° C.

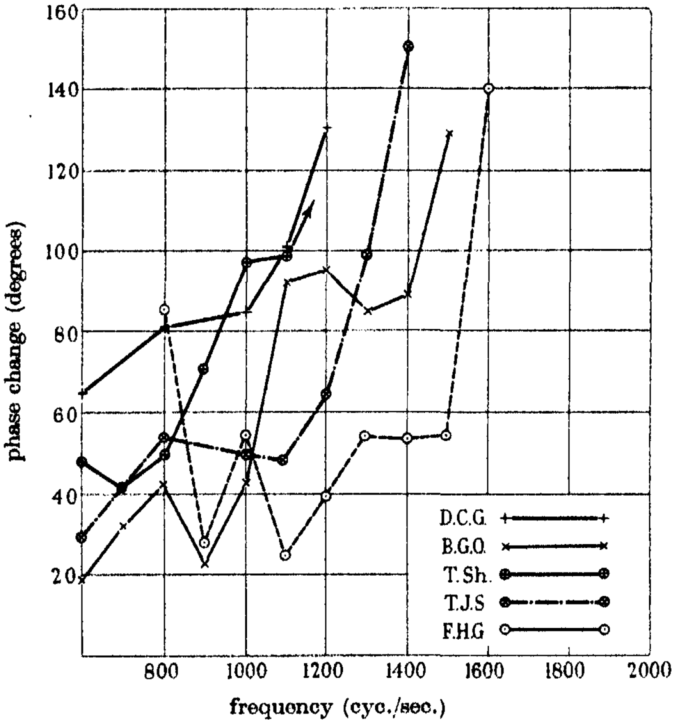

FIGURE 2

Minimum refractory periods of 0·8–0·9σ for the bullfrog, and 0·9–1·1σ for the turtle were recorded by Erlanger, Gasser and Bishop (1927), while for a *Plantaris* preparation Blair and Erlanger (1933) found values of about 0·9–1·1σ at 24° C. Lorente de Nó (1935) measured refractory periods for the motoneurone as short as 0·6σ. It is to be noted that in these later researches the effect of previous shocks was investigated, and that they show conclusively that such previous stimulation lengthens the refractory period to a marked degree. Thus in the case of the auditory nerve, with its normal stimulation in successive cycles of sound waves, the effective refractory period might be expected to exceed the values found in experiments expressly designed to exclude this effect of repeated stimuli. The period of about 1σ suggested by the present work falls therefore well within the range of values which previous observations have given for nerves in general.

For the auditory nerve itself similar evidence is given in another way by work at Harvard on the electrical phenomena of this nerve in the cat. It has been shown (e.g. Hallowell Davis, Derbyshire and Saul 1933; Hallowell Davis 1935; Derbyshire and Davis 1935) that the amplitude of the action potentials recorded from the auditory nerve remains virtually independent of frequency between 500 cyc./sec. and 800 cyc./sec., but a little above the latter frequency falls sharply to about half the previous value. The potential remains at this new level with further rise of frequency up to about 1600 cyc./sec., where there is a second sharp drop to about one-third of the original micro-voltage. Davis interprets these phenomena as due to failure of the nerve fibres to respond in each cycle when the period of the sound waves approaches the absolute refractory period. Above this critical frequency each fibre can only fire in alternate cycles, and above frequencies twice that corresponding to the refractory period only once in every three cycles. The first step down in action potential at 800 or 900 cyc./sec. thus indicates a refractory period of 1·1 or 1·2σ in the case of the cat. A few experiments seem to indicate a critical frequency of about 1000 cyc./sec. or a refractory period of 1·0σ.

The present paper thus suggests that the refractory period of the human auditory nerve is of the same order, probably rather less than 1 msec.

The author wishes to thank the Medical Research Council for grants which have made the work possible, and to express his appreciation of the suggestions made by Dr J. H. Shaxby, in whose laboratory the experiments were performed.

SUMMARY

The least perceptible changes of phase in binaural localization (measured from zero phase difference) have been determined for several observers at various frequencies in an endeavour to find if any upper frequency limit for the detection of such changes existed. Pure tones were used in the experiments, and the changes of phase were made by an electrical method. It is shown that phase (time) difference alone, with no intensity difference at the two ears, can serve as the basis of localization. The results show that for nearly all observers the detection of changes of phase becomes difficult

at about 1300 cyc./sec., and impossible at about 1500 cyc./sec. This indicates that the refractory period for the human auditory nerve is about 1σ, and therefore of the same order as in other types of nerve.

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The Egyptian exploration of the Red Sea

BY A. F. MOHAMED

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The success achieved by the John Murray Expedition to the Indian Ocean, 1933–4, in the Egyptian research ship *Mabahiss* aroused the interest of Egypt in oceanographic exploration. When the *Mabahiss* returned to Alexandria in May 1934, the University at Cairo had under consideration a plan for sending out an expedition to explore the whole area of the Red Sea in 1935–6. Meanwhile, it was thought advisable to send a preliminary expedition, the experience of which might be a useful guide for the 1935–6 expedition,* to its northern regions.

This preliminary expedition, which lasted from 18 December 1934 to 20 February 1935, marks the first attempt by modern Egypt to undertake systematic oceanographic research. A note on the topographical and biological results of this expedition has already appeared (Crossland 1936). The object of the present communication is to report some of its hydrographic and chemical results which are believed to have increased our knowledge of the oceanography of the Red Sea.

PREVIOUS INVESTIGATIONS

The basis of our present-day knowledge of the oceanography of the Red Sea is largely provided by the results of the Austrian Expedition on the *Pola* in 1895–6 and 1897–8 (Luksch 1898, 1901; Natterer 1898, 1901). However, oceanographic instruments and methods were at that time imperfect, and the *Pola* observations therefore lack the accuracy desirable in modern oceanography. More recently, two Italian Expeditions on board the *Magnaghi* carried out an extensive programme of oceanographic investigations in the Red Sea in 1923–4 and 1929 (Picotti 1927; Vercelli 1925, 1927, 1931). The results of these investigations have in general amplified our knowledge of the physical oceanography of the Red Sea and have in particular thrown abundant light on the regime of currents in the Straits of

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* Owing to the situation created by the Italo-Abyssinian conflict the Egyptian Government was forced to postpone the 1935–6 expedition; financial difficulties thereafter caused further delay. It is earnestly hoped that it will be soon possible to send out such an expedition.

Bab-el-Mandeb as well as on the tides and currents in the Gulf of Suez. Unfortunately, not only were these investigations restricted to certain regions but they did not deal sufficiently with the chemistry of the Red Sea waters. Finally, we have the observations of the Snellius Expedition in 1929 (Van Riel 1932) and of the John Murray Expedition in 1933–4 (Sewell 1935), which were made at but a few stations in the southern half of the sea.

THE RED SEA WATERS

(1) *Hydrography*

One of the main objects of the Egyptian exploration of the Red Sea is to secure, through the modern development of hydrographic instruments and methods, systematic oceanographic observations at a series of standard levels from the sea surface down to the bottom. The importance of such exact data for a better understanding of the properties and movements of the Red Sea waters cannot be over-emphasized.

During the cruises of the *Mabahiss* in the winter of 1934–5 serial determinations of temperature and halogen content were systematically made along ten sections at some forty-five stations distributed over the Gulf of Aqaba, the Gulf of Suez, and the adjacent regions of the Red Sea. The area investigated, as well as the position of the various sections, is shown in figure 1.

The Gulf of Aqaba, a deep trough with depths between 1650 and 1830 m. near its centre, is separated from the deep parts of the Red Sea by a shallow sill in the Strait of Tiran. For the first time our temperature and salinity observations in this strait have brought to light the mode of the exchange of water masses between the Gulf of Aqaba and the Red Sea, a matter which did not receive the attention of former expeditions. The vertical distribution of the potential values of temperature along section E (figure 2) indicates that there passed, at the time of observation, through the Strait of Tiran two currents, a surface current from the Red Sea into the Gulf of Aqaba and a bottom current out of the gulf, the latter contributing to the formation of the Red Sea bottom water.

An exactly similar picture is given by the vertical distribution of salinity. It is noteworthy that these movements are analogous to those through the Straits of Bab-el-Mandeb in the winter season.

In the Gulf of Aqaba itself our temperature observations have, for the first time, revealed the presence of an adiabatic increase in the temperature of the deep water; the temperature, after reaching a minimum of *ca.* 21·20° C at a depth between 300 and 400 m., increases slowly and steadily towards the bottom. The observed gradient of this increase amounts to 0·20° C/1000 m. increase of depth, which is in complete accord with the magnitude of the adiabatic effect to be expected on theoretical grounds. The observation of this adiabatic heating at great depths in the Gulf of Aqaba escaped completely the investigations of the *Pola* through lack of precision in the thermometers then in use, which read to $\frac{1}{10}$° C only.

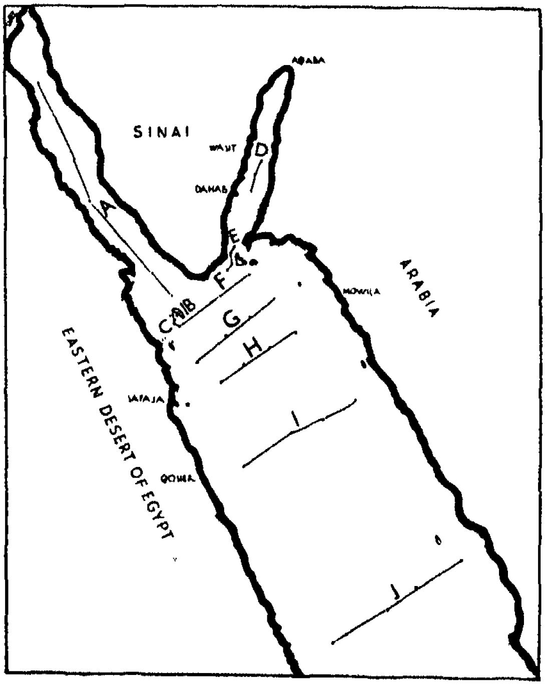

FIGURE 1. Area of investigation.

The precise variation of temperature with depth in the shallow Gulf of Suez varies from one region to another as shown by its vertical distribution along the medial line of the gulf (figure 3), which reveals the progressive arrangement of the isothermal pattern as well as the complexity of the thermal regime. A more or less similar picture is given by the distribution of salinity (figure 4); and both pictures suggest the inflow of a warm, less saline surface current from the Red Sea into the gulf and the outflow of a bottom current, more saline and less warm. The efflux of the latter into the Red Sea, on both sides of Shadwan Island, was clearly indicated by our temperature and salinity observations in the southern approaches of the

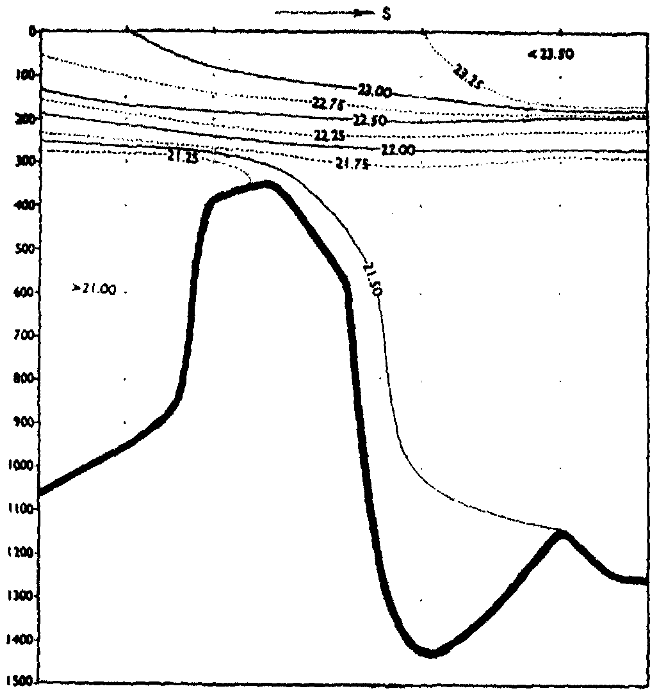

FIGURE 2. The vertical distribution of the potential values of temperature along section E.

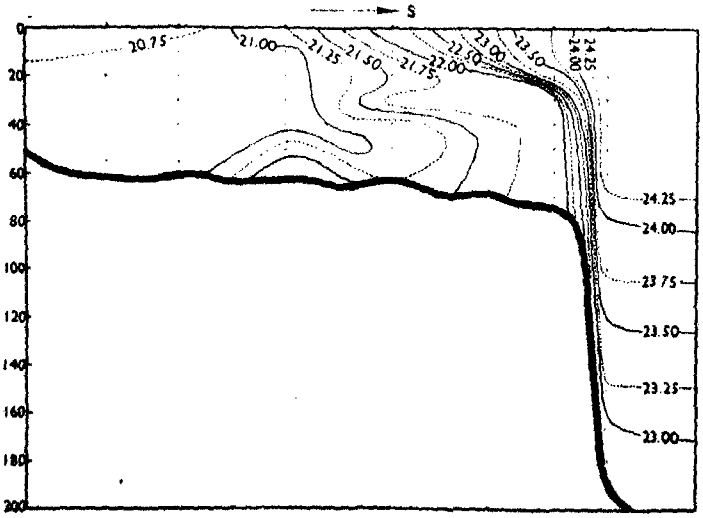

FIGURE 3. The vertical distribution of temperature along section A.

Gulf of Suez. Moreover, the analysis of the correlation curves of these observations has suggested that the water of this bottom current mixes at the edge of the gulf with the water of the upper strata in the Red Sea, the resulting admixture sinking down and contributing to the formation of the Red Sea bottom water. A further light is thus thrown on the origin of the bottom water in the northern Red Sea.

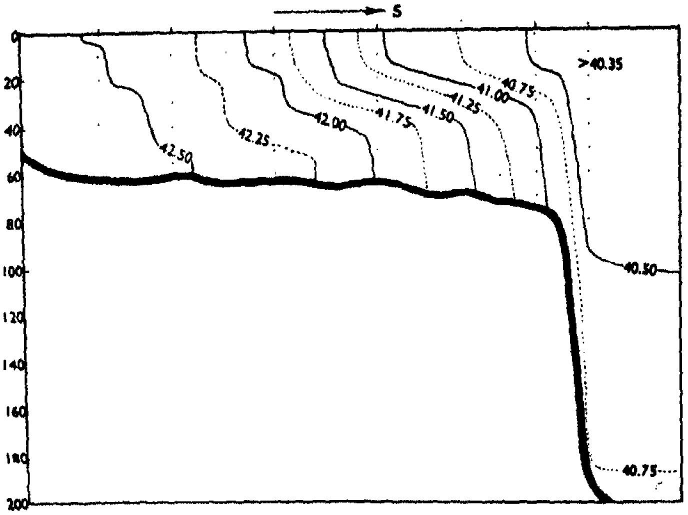

FIGURE 4. The vertical distribution of salinity along section A.

The variation of temperature with depth in the Red Sea proper agrees, in its characteristic features, with that in the Gulf of Aqaba. From its high values in the upper stratum, the temperature decreases to a minimum value of *ca.* 21·60° C (i.e. about 0·50° C higher than the corresponding minimum value in the Gulf of Aqaba) at a depth of 500–600 m., below which it increases adiabatically with increase of depth. This adiabatic heating at great depths in the Red Sea proper was first observed by the Italian Expedition in 1923–4 (Vercelli 1927). The vertical distributions of temperature and salinity in the northern regions of the Red Sea are illustrated in figures 5 and 6.

According to the *Pola* observations the temperature of the surface waters in the Red Sea shows a regular increase, and their salinity a similar decrease, from west to east. This regular distribution led Luksch (1901) to formulate his hypothesis of cyclonic circulation in the Red Sea, with a warm, less saline current, moving north along the Arabian coast, and a more saline, less warm current, moving south along the African coast. The ***Magnaghi***

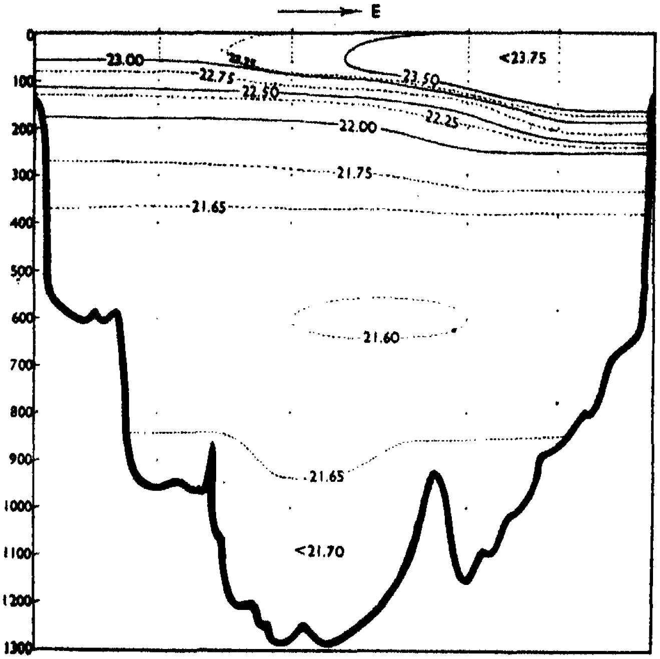

FIGURE 5. The vertical distribution of temperature along section H.

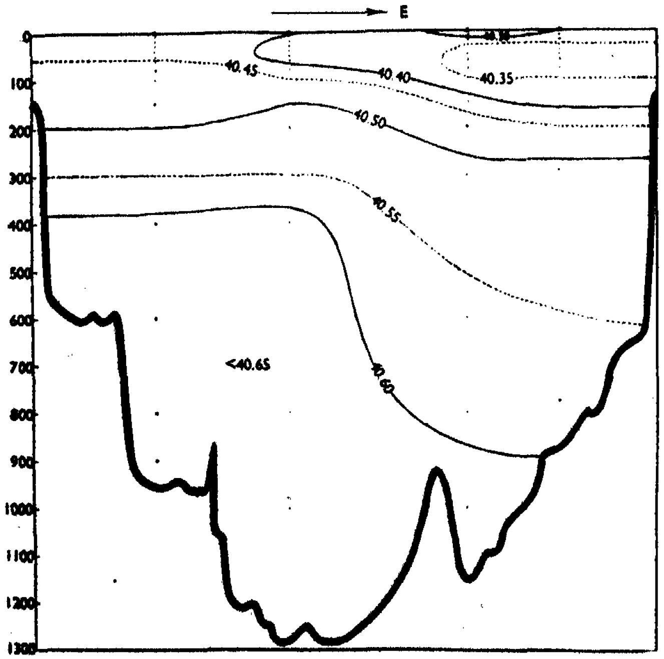

FIGURE 6. The vertical distribution of salinity along section H.

observations in the southern regions have, on the other hand, suggested that such a regular distribution does not constitute a permanent feature of the hydrography of the Red Sea (Vercelli 1927). Moreover, the direct current measurements made by the first Italian Expedition refuted Luksch's hypothesis in the southern regions (Vercelli 1925), and its validity in the central and northern regions was questioned by Vercelli (1927). Nevertheless, our observations give a clear indication of such a regular distribution in the northern regions, though, along some sections, certain factors upset its regularity. This orderly distribution does not, however, necessitate our falling back on Luksch's hypothesis, for it lends itself most readily, as will be shown elsewhere, to interpretation on meteorological grounds.

The temperature and salinity observations were further treated according to the dynamic method of Bjerknes, and velocity profiles of the currents normal to sections G, H, I and J were thus constructed. A striking feature of these profiles is the alternation in the direction of flow across each section, a feature which has suggested the existence of vortex movements. There is evidence to support this view in the abnormal low values of temperature, which were observed at the surface at certain stations, as well as in the abnormal high values, which were found much below the surface at certain others. The cause of these abnormal conditions could not be traced to any obvious source, and can be ascribed to cyclonic and anticyclonic vortices, the former causing cold water to well up from below to the surface and the latter resulting in the warm surface water sinking down. For this and other reasons, the system of currents in the Red Sea cannot be described, on the basis of our observations, so simply as Luksch formulated it; rather it is complex and calls for further investigations for its elucidation.

(2) *Chemistry*

On board the *Mabahiss*, determinations of dissolved oxygen were systematically made at all hydrographic stations, *p*H values at most of them and phosphate content at a good number.

Our oxygen observations provide for the first time ample information for a detailed study of the distribution of oxygen in the Red Sea. As a result, the oxygen-depth curve for the Red Sea proper can be distinguished into four zones, characterized by differences in oxygen content: (*a*) a zone of high oxygen content at the surface; (*b*) a peak of maximum oxygen near the surface, the so-called photosynthetic zone; (*c*) a mid zone, in which the oxygen content decreases rapidly with increase of depth until it reaches a minimum concentration in the 400–600 m. stratum; and (*d*) a deep zone in which the oxygen content increases progressively with depth.

Of these zones, the minimum oxygen stratum existing at mid depths has recently received great attention, and, consequently, much information has been accumulated to show its existence in several oceanic regions. Its presence in the Red Sea passed unnoticed by the Austrian Expedition, whose programme included the analysis, at each hydrographic station, of only two samples for oxygen, the one obtained from a depth of 100 m. and the other from the vicinity of the bottom. It remained for the Snellius Expedition (Van Riel 1932) and the John Murray Expedition (unpublished data) to reveal its presence in the southern region of the Red Sea; and now our observations establish its wide existence in its northern region. The distribution of oxygen in this latter region is shown in figure 7.

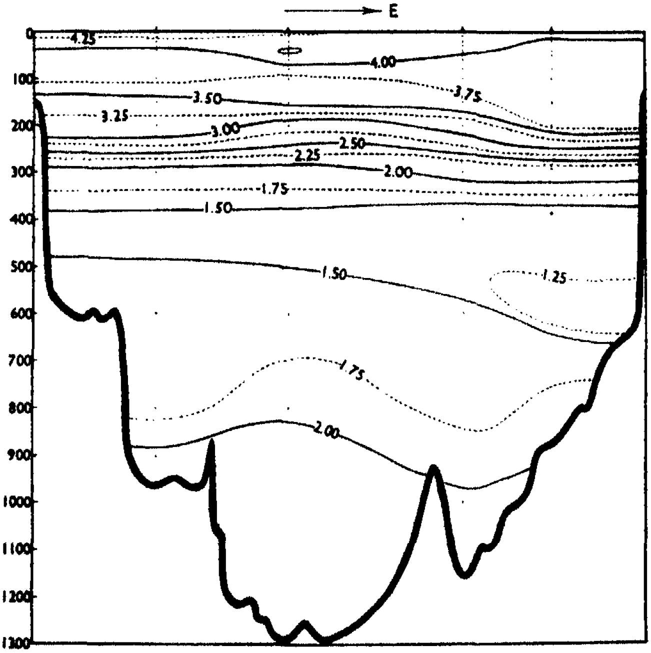

FIGURE 7. The vertical distribution of oxygen (c.c./l.) along section H.

The vertical distribution of oxygen in the Gulf of Aqaba presents two striking differences. In the first place, the minimum oxygen mid stratum is non-existent, the oxygen content decreasing continually with increase of depth; and, secondly, the oxygen content of the deep water is much higher than in the Red Sea, a point of difference which was found also by Natterer (1898). In the shallow Gulf of Suez, the oxygen content is generally high throughout the water column and shows but slight variations with depth.

The pH of the Red Sea waters was found to vary from 8·02 to 8·10 at the surface. Its variation with depth is in many respects similar to that of oxygen; for instance, there is a minimum pH layer at intermediate depths of 300–600 m. in the Red Sea proper.

Much information on the distribution of phosphate in several oceanic regions has of late years become available, but no phosphate observations from the Red Sea existed prior to the Egyptian Expedition. The phosphorus content, present as inorganic phosphate, of its waters is now brought to light by our observations. In the Red Sea proper the phosphate content varies from 0 to 6 mg. P per cu. m. in the surface layer, below which it increases to a maximum concentration of 64–69 mg. P per cu. m. at intermediate depths, usually between 400 and 600 m. With increasing depth the phosphate decreases down to 800 m., below which a constant concentration (34–39 mg. P per cu. m.) is usually present.

The phosphate content in the Gulf of Aqaba also increases until a depth of 700 m. is reached; below this the concentration does not show any significant variation with depth, but remains relatively constant at *ca.* 23 mg. P per cu. m., a concentration which is much lower than that of the deep water in the Red Sea. It is thus evident that whereas the deep water in the Gulf of Aqaba is impoverished in phosphate, that in the Red Sea contains as much as that of any oceanic region.

THE RED SEA BOTTOM DEPOSITS

Another object of the Egyptian exploration of the Red Sea is to study the nature and distribution of its bottom deposits. Our knowledge of these deposits, derived from the investigation of the *Pola* samples (Natterer 1898), is very generalized and leaves much to be desired.

An investigation of thirty-eight bottom samples, collected by the Egyptian Expedition from the northern Red Sea, was undertaken. Three of these samples were obtained from the Gulf of Suez, five from the Gulf of Aqaba, and the rest from the adjacent regions of the Red Sea. In the course of this investigation the carbonate, organic carbon, and nitrogen content of these sediments were determined; the major chemical constituents of a good number of them were also estimated.

The results of this investigation show that these sediments are very rich in carbonates, those from the Red Sea proper being richer than those from either the Gulf of Suez or the Gulf of Aqaba. The carbonate content, calculated as calcium carbonate, was found to vary from 42·20 to 96·96 %, the lowest value being found in a sample from 1113 m. in the Gulf of Aqaba and the highest in one from a depth of 18 m. in the vicinity of the western reefs in the Red Sea. In general, the carbonate content does not show any definite correlation with depth of water, although there is sufficient evidence to indicate that it falls, in the Red Sea proper, with increase of depth and with increase of distance from the coast.

It has been found that the organic matter present in these sediments, as measured by the nitrogen content, does not exceed 1 %, and therefore is very low compared with that of other marine sediments. The highest content of organic matter was found in those sediments obtained from the southern approaches of the Gulf of Suez (nitrogen content = 0·082 to 0·091 %), where organic residues transported by the outgoing current are likely to accumulate. In the Gulf of Suez itself the content is relatively high (nitrogen content = 0·05 %); on the other hand, it is remarkably low in the Gulf of Aqaba, the nitrogen content being 0·025 % or less. In the Red Sea proper it varies widely, and is generally high in those sediments collected from the shallow coastal regions and low in those secured from great depths. The carbon/nitrogen ratio, which affords a further means for estimating organic matter, is lower than that of other marine sediments; its average in the Gulf of Suez is 7·7, in the Red Sea 4·6 and in the Gulf of Aqaba 4·0. On the whole. the richer the sediment is in organic matter, the higher is its C/N ratio.

With regard to the major chemical constituents of these sediments the following data are available. It is found that the silica content varies from 13·74 to 36·32 % in the Gulf of Aqaba, from 14·07 to 23·33 % in the Gulf of Suez, and from 2·86 to 12·04 % in the Red Sea proper. It is of interest to note that the lowest value was found in a sample procured from the slope of a submarine hill lying west of Shadwan Island. The alumina content, which varies from 1·21 to 11·95 %, is directly proportional to that of silica. Furthermore, it is found that the total iron content (0·74 to 3·11 %, calculated as ferric oxide) is highest in the Gulf of Suez and lowest in the Red Sea; but it is fairly high in the Gulf of Aqaba. It is also shown that the magnesia content (2·30 to 3·43 %) does not vary widely, while the lime content corresponds well with the results of the carbonate analysis. Finally, it is found that the hygroscopic water retained by these sediments does not exceed 1 %.

On the whole, the results of this chemical investigation lead to the conclusion that the environmental conditions of deposition are different in each of the three main regions investigated.

This work was carried out partly on board H.E.M.S. ***Mabahiss*** in the Red Sea and partly at the Department of Oceanography, University of Liverpool.

SUMMARY

An account is given of some of the more outstanding results of a preliminary Egyptian Expedition sent to the northern Red Sea in the winter of 1934–5. The important features of the hydrography of the area investigated, such as the origin of bottom water in the northern Red Sea, the water movements through the Strait of Tiran, the adiabatic temperature changes at great depths in the Gulf of Aqaba, the thermal regime of the Gulf of Suez and the nature of circulation in the Red Sea, are described. The chemical observations have revealed the presence of a maximum phosphate layer at intermediate depths in the Red Sea; but at these depths the oxygen concentration as well as the pH is at its minimum. The analyses of thirty-eight bottom samples collected from the northern Red Sea and its adjoining gulfs suggest that the environmental conditions of sedimentation are different in each of the three main regions investigated.

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The pigmentary effector system
IX. The receptor fields of the teleostean visual response

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1. INTRODUCTION

Both among vertebrates and among Crustacea one commonly meets with two co-existent modes of chromatic response to photic stimulation. One is the *dispersion* ("expansion") of melanophores and certain other chromatophores under the local (primary) influence of light on the skin. The other is *aggregation* ("contraction") of melanophores and of certain other chromatophores when light reflected from the surroundings impinges on the organs of vision, in contradistinction to *dispersion* ("expansion") when only *overhead* illumination strikes the eye. Though the primary (local) response is usually subordinate to and is more or less overruled by the secondary or visual response, the relative importance of the two components varies within wide limits. In particular species either may be negligible in comparison with the other.

When, as more commonly, both contribute significantly to the observed result, a blinded animal is necessarily more pale in darkness than in light. Probably this fact influenced all the earlier investigators who, including the senior author (1924), paid little attention to the otherwise paradoxical fact that animals kept on a "black background" (i.e. under conditions of overhead illumination in light absorbing surroundings) are much darker than animals kept in similar conditions with no light at all. Subsequent analysis of the normal course of colour change, both in vertebrates and in Crustacea, has shown that this is also true of species which have no appreciable primary response, and that the difference generally exceeds the limits of variation consistent with the co-existence of a detectable primary response. It is therefore clear that the difference between the "white background" response and the "black background" response is not due to intensity alone.

The fact that it is not an intensity effect leads us to ask whether it is due to spatial differences among the receptive elements in the visual organs or of their nervous connexions. Hogben and Slome (1936) have shown that the difference between white and black background responses of Amphibia depends on the orientation of retinal elements which are intrinsically different. Overhead illumination in a black container involves stimulation of a restricted area of the floor of the retina by rays compressed in a cone of which the half-angle is the *critical* angle for air and water. This area is the specific *receptor field* of the black background response. The experiments of Hogben and Slome were based on a species (*Xenopus laevis*) with eyes directed upwards. By a different class of experiments on a species (*Fundulus heteroclitus*) with lateral eyes Butcher (1938) has since demonstrated differentiation of receptive fields for the black and white background responses of fishes. Butcher was also able to detect morphological differences in the ventral and the dorsal portions which respectively *include* the receptor fields for the black and white background responses. Smith (1938) has published evidence pointing to the same general conclusions with reference to chromatic behaviour in Crustacea (*Ligia*).

While previous work sufficiently demonstrates the dual character of the receptive field involved in the secondary response, experiments on vertebrate types carried out so far have not supplied the necessary data for prescribing the precise limits of one or the other. Such data necessarily include more information about the optical properties of the eyes of the lower vertebrates than is at present available.

The present enquiry was first undertaken to clarify this issue. The experiments recorded in this communication deal with the stickleback (*Gasterosteus aculeatus* Linn.). The most straightforward way of delimiting the receptor fields for the black and white background responses is to compare response to overhead and inferior illumination in containers of various sorts. The possibility that a given area of the retina is neutral from this standpoint implies that the animal behaves as it would behave in darkness when illumination is restricted to such a region. This presupposes the possibility of distinguishing clearly between normal chromatic behaviour in darkness and the white or black background responses to overhead illumination.

Among Amphibia the equilibrium condition of the dermal melanophores in darkness is usually midway between the extremes for white or black background equilibrium with overhead illumination. Among bony fishes it may be indistinguishable from the white background response. Hence the terms "pale" and "dark" phase used by some contemporaries are highly misleading. To discriminate clearly between the white background response and the equilibrium conditions of fish melanophores, it is essential to describe the *microscopic* phenomena of colour change with the greatest practicable precision and to discriminate between the process of reaching equilibrium in different conditions of illumination. Quantitative methods for recording the behaviour of pigmentary effectors have been proposed by Hogben and Slome (1931), by Hill, Parkinson and Solandt (1935) and by Wykes (1937). Objections to the last two are fully discussed by Neill in a communication which will shortly be published from this laboratory. It is therefore unnecessary to discuss them in this context. The melanophore index (M.I.) of Hogben and Slome is used in the tables set forth below.

2. ESSENTIAL PRECAUTIONS IN OBSERVATIONS ON FISH MELANOPHORES

It will be subsequently shown that *Gasterosteus* has no primary response which is detectable on the M.I. scale. The equilibrium values for photic responses are:

Darkness	2·1
Overhead illumination with white background	1·3
Overhead illumination with black background	4·7

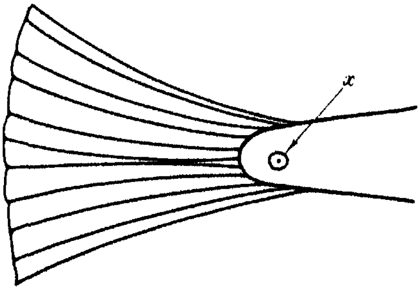

FIGURE 1

Thus the melanophores of sticklebacks kept in darkness and the melanophores of sticklebacks kept with overhead illumination in a white container would both be described as "contracted" by workers who record their observations in this form. It is therefore advantageous to have a second criterion for discriminating between the two. It will now be shown that the time graph gives a decisive one.

In what follows all figures for the M.I. were based on melanophores of approximately the same situation (x in figure 1). Initial attempts to determine the time graph of normal response showed that mechanical contact inhibits colour change in *Gasterosteus*. This could be recognized macroscopically. Microscopic observations on individual fish at varying intervals after removal from their containers for examination yielded the following mean figures for the melanophore index:

	7	30	60	120 (sec.)
From black background	4·7	4·3	3·8	3·7
From white background	1·3	1·8	2·3	2·3

With the exception of the one shown in figure 2 all time graphs subsequently recorded were therefore based on the average of six to twelve individuals which had not been previously recorded during the course of the experiment. The time graphs in figure 2 show the distortion which would have resulted if this precaution had not been taken.

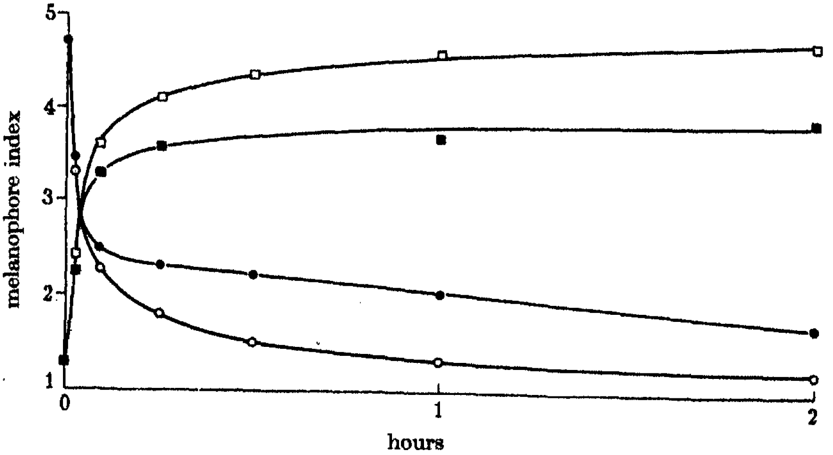

Figure 2. Time graphs of transition from white to black background equilibrium and vice versa with overhead illumination. Each white square and each white circle is based on the mean melanophore index of thirty fish recorded *on that occasion only* and then discarded. Each black circle represents the mean figure for one and the same group of ten fishes recorded *successively*. Each black square represents the mean figure for another group recorded successively.

Owing to the rapidity of the initial stages of background response it is essential to make each determination of the M.I. as quickly as possible. A satisfactory device which keeps the time taken after a little practice within 5 sec. is to keep each fish in a separate tube immersed in the container. The tubes (figure 3) used were constructed with a large hole through which the fish could be introduced, and at each end, a flat hole sufficiently wide to allow the tail of the fish to project from it on to the microscope stage during observation. In the tubes used fish under 1¼ in. long respond normally. All fishes used in this investigation were between ¾ and 1 in. long.

Another necessary precaution in making reliable observations is proper control of temperature. In our experience 12° C proved to be the optimum for background response. A sudden deviation of more than 2° C on either side profoundly affects the chromatic behaviour of *Gasterosteus*. All our recorded experiments were therefore carried out with thermostatic control at 12 ± 1° C. Chromatic behaviour of sticklebacks is also influenced by pollution. In experiments of long duration (e.g. those recorded in figure 6) the fish were kept in running water by the arrangement shown in figure 4.

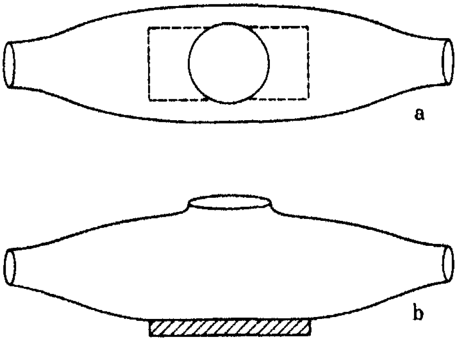

FIGURE 3. (*a*) seen from above, (*b*) side-view.

3. TIME RELATIONS OF CHROMATIC BEHAVIOUR IN *GASTEROSTEUS*

The importance of studying time relations has already been emphasized by one author (L. H.) in connexion with the analysis of the co-ordinating machinery of colour change. In any behaviour-isolate the period which elapses between the application of a stimulus and the maximum response is made up of three components: (*a*) the latent period of the receptor; (*b*) the rate of transmission in the co-ordinating system; and (*c*) the reaction time of the effector. The few available figures for (*c*) based on response to hormones, to other reagents in perfusion experiments or to local electrical stimulation, show that pigmentary effector organs react very slowly. So in general (*a*) and (*b*) are always negligible compared with (*c*) if co-ordination is wholly nervous. If the time taken to reach equilibrium is greater than one hour we may generally infer the intervention of chemical co-ordination. The reaction time of the melanophores of *Gasterosteus* is extremely rapid in comparison with that of the Amphibian type. Complete contraction of fully expanded melanophores of *Gasterosteus* can be brought about by the action of adrenalin within 3 min. This means that, if the co-ordination of colour change in *Gasterosteus* is exclusively nervous, equilibrium should be accomplished within a period not exceeding 5 min.

Within wide limits the intensity of illumination has no effect on the rapidity of response to background reversal. No significant deviations at corresponding intervals are seen in table 1, in which each mean figure is based on a separate batch of ten fish which had not been handled since the beginning of the experiment. The mean figures of all three intensity groups have therefore been combined to draft the standard (i.e. the uppermost and lowermost) time graphs shown in figure 2.

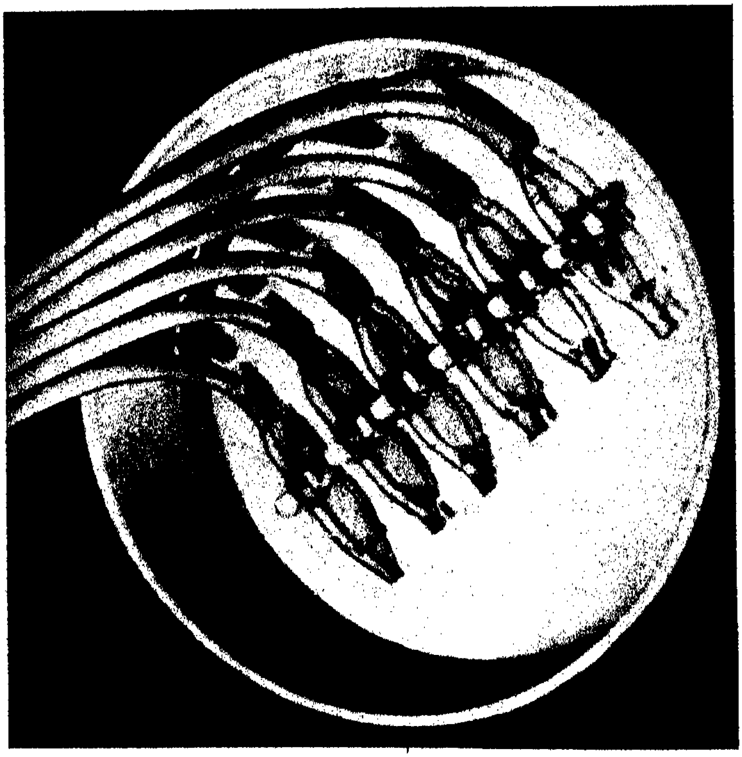

FIGURE 4

The fact that readings of the M.I. over such a wide range of intensity reveal no significant differences indicates that any *primary* response of *Gasterosteus* is too small to be detected by the method used. This means that the eyeless fish exposed to daylight should have the same M.I. as the

TABLE 1

Each figure based on 10 fish

Time min.	Daylight	Tungsten lamp 300 ft.-candles	Tungsten lamp 0·5 ft.-candles	Daylight	Tungsten lamp 300 ft.-candles	Tungsten lamp 0·5 ft.-candles
0	4·7	4·6	4·7	1·3	1·3	1·25
1	3·5	3·5	3·6	2·25	2·2	2·1
3	2·7	2·8	2·9	3·15	3·0	3·1
15	1·8	2·0	2·0	4·1	4·1	4·2
30	1·5	1·7	1·8	4·35	4·4	4·3
60	1·3	1·4	1·5	4·6	4·6	4·5

normal fish kept in darkness. Presumably on account of bleeding from the orbital sinuses, fish usually die soon after ophthalmectomy. For that reason we are unable to present figures based on animals which had survived till degeneration of the optic nerves was complete. For what they are worth the following data are consonant with the conclusion stated. They refer to fish put in a black container illuminated with a 100 W. lamp at 6 ft. The sticklebacks were anaesthetized with urethane during the operation which was performed in two stages. To reduce shock and mortality from loss of blood the second eye was removed 2 days after the first.

Hours after complete removal	No. of fish	Average M.I.
24	12	2·1
48	7	2·0
72	4	2·1

Since equilibrium is reached only after the lapse of a period sufficiently long to produce results due to pollution, the experiment summarized in figure 6 was carried out with the precautions shown in figure 4. The time relations of normal response to overhead illumination are set forth in the next two figures (figures 5, 6). The time graphs of colour changes in *Gasterosteus* exhibit two noteworthy features:

(*a*) Though the initial stages of background response are rapid the final stages are prolonged (figure 5).

(*b*) The protracted transition to equilibrium in darkness (figure 6) is of a different order of magnitude from the background response.

These features of the time relations of colour change in *Gasterosteus* are significant in connexion with the evolutionary view of chromatic behaviour put forward by Hogben and his associates on the basis of their work on *Xenopus laevis*, the chameleon and various species of Elasmobranch fishes.

According to this view we must distinguish between two essentially different aspects of colour change:

(*a*) What happens when an animal is brought out of darkness into light and vice versa?

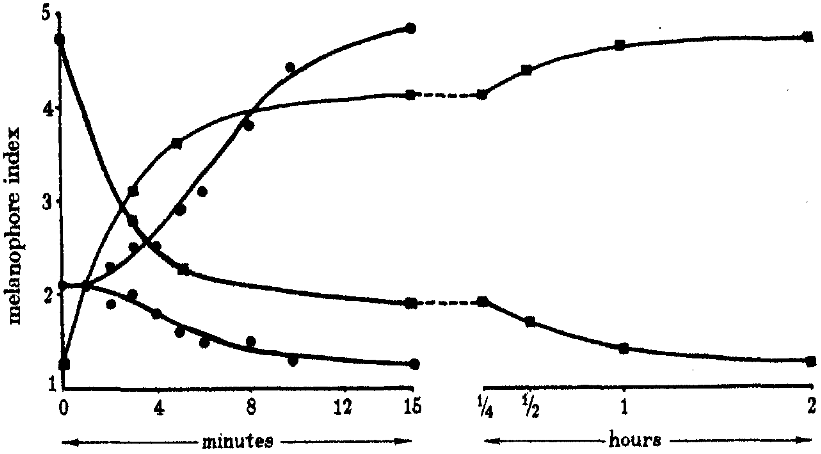

FIGURE 5

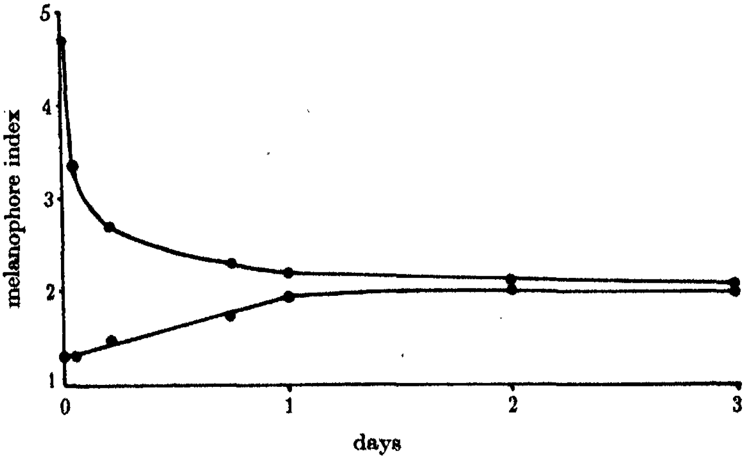

FIGURE 6

(*b*) What happens when an animal is transferred from overhead illumination in light *reflecting* to overhead illumination in light *absorbing* surroundings and vice versa?

A hypothesis which brings all the data hitherto recorded into line is that in vertebrates generally (*a*) results from secretion or excretion of the

B hormone of the *pars intermedia* of the pituitary gland, and that (*b*) results *either* from the secretion (or excretion) of a second hormone (*W*) located in the *pars tuberalis* (or in some other gland controlled by it), *or* from direct nervous control *secondarily* superimposed upon, and to a greater or less extent replacing, a more archaic humoral mechanism. This eclectic view which receives fresh support from enquiries by Neill and by Waring (1939), on the chromatic behaviour of the eel in this laboratory is opposed by Parker and his colleagues who do not contest the recorded evidence derived from the intensive study of *Xenopus*, but prefer to adopt an *ad hoc* view of chromatic behaviour in the species which they have studied.

The difference of outlook which at present separates the two schools of workers on colour change arises partly from the fact that Parker and his colleagues distinguish only two phases, the "dark" and the "pale" and have refrained from pursuing their enquiries into the respective time relations of the white-black background and the light-darkness changes. Of the two features of our time graphs stated above, the first, which could not be detected by the descriptive methods which Parker and his colleagues continue to use, is consistent with the view that the white background response is brought about by a peripheral nervous, superimposed on a more archaic humoral, control which reinforces it in the final stages. The second conclusively shows that the co-ordinating mechanism responsible for the transition from light to darkness is different from the co-ordinating mechanism responsible for background interchange.

Several investigators have shown that some Teleostei do not respond to the action of pituitrin, i.e. to *B* containing extracts when it is injected in the "pale phase"; and this is apparently felt to be an insuperable objection to a generalized hypothesis such as stated above. Since observers who have recorded negative results of this kind have invariably used animals which have been made pale by transference to a white background in daylight, the experiment has no relevance to the issue. The hypothesis put forward in the foregoing remarks implies that in species which have acquired nervous control, the latter can override the action of *B* which is liberated into the circulation when animals are brought out of darkness into light. *Ex hypothesi* nervous stimulation resulting from exposure to light on a white background therefore antagonizes the action of *B*. Hence we need not expect *B* to act on animals exposed to a white background in light. On the other hand we should expect that extracts containing *B* would antagonize the pallor induced by keeping fish *in darkness*.

From the standpoint of the present investigation the striking difference between the time graph of the pallor which ensues in total darkness and the time graph of pallor which results from transference to a white from a black background with overhead illumination gives us a new and more delicate means of discriminating between the two physiological processes which Parker calls the *pale phase*. With this additional information at our disposal we can confidently distinguish between regions of the retina which are neutral and regions of the retina which initiate the white background response when stimulated.

4. THE OPTICAL SYSTEM OF THE EYE OF *GASTEROSTEUS*

In his recent investigation of retinal orientation with respect to chromatic behaviour of *Fundulus*, Butcher (1938) has recorded the effect of superior and inferior illumination when the upper half or the lower half of the eyes are masked. This procedure is not necessary for the present purpose and cannot give precise information concerning the limits of the regions concerned with the background responses. As already pointed out by Hogben and Slome (1936), the limits of incident radiation which enters the eye of an aquatic species when no light is scattered or reflected from the walls of the container itself, are defined *by the cone whose half angle is the critical angle for water, i.e.* 49°. To define limits of stimulation in the situations described in Butcher's enquiry it is therefore sufficient to know: (*a*) the extent to which incident rays are refracted by the optical system of the eye itself, and (*b*) the relative dimensions of its constituent parts. Before describing our own experiments on the effect of changing the direction of incident light, we shall therefore set forth our observations on the dimensions and on the optical properties of the constituent parts of the eye of the stickleback.

Dimensions. The eye of a teleostean fish differs from that of a land vertebrate both with respect to its dimensions and to its structural characteristics. Two important structural differences are: (*a*) the absence of ciliary muscles, (*b*) paucity of contractile elements in the iris. Our own histological examination did not reveal the presence of clearly defined muscle fibres in the latter. Injection of 5% pilocarpine or 5% atropine beneath the cornea and direct electrical stimulation of the iris alike failed to produce any detectable change in the size of the pupil.

The principal dimensional characteristics which distinguish the Teleostean eye from that of a Tetrapod are: (i) the sphericity of the lens and (ii) the great lateral compression of the eye as a whole about the axis passing through the blind spot, lens and cornea. With regard to the sphericity of the lens two Vernier microscope measurements made on diameters at right angles may be cited as typical. The mean radius of the first was 0·7275 ± 0·0275, i.e. a deviation of 3½ % from the mean value for rectangular radii. This lens had been allowed to dry slightly. A second, kept moist throughout, gave the figure 0·83 ± 0·01, i.e. the deviation of two radii at right angles was 1½ % of the mean value. From the standpoint of geometrical optics we may therefore treat the lens of the eye of *Gasterosteus* as a perfect sphere without introducing errors of an order exceeding the accuracy of our micrometer estimates. The radius of curvature of the cornea determined by measurement of the depth of the pupil and width of the iris of an eye with pupil aperture 1·9 mm. and lens of 0·83 mm. was 1·8 mm., i.e. the radius of curvature of the cornea and lens may be taken as approximately 2·2 : 1.

The scale diagram of the eye shown in figure 7 is based partly on microscopic sections and partly on micrometer measurements of the living eye. The figures summarized below (table 2) are representative:

TABLE 2

	A	B
(*a*) Vertical diameter of whole eye	3·76	4·71
(*b*) Longitudinal diameter of whole eye	3·76	4·75
(*c*) Vertical diameter of iris	1·50	2·02
(*d*) Longitudinal diameter of iris	1·81	2·40
(*e*) Horizontal diameter of whole eye	2·69	3·35
(*f*) Diameter of lens	1·41	1·81
(*g*) $e \div \frac{1}{2}(a+b)$	0·715	0·708
(*h*) $e \div f$	1·91	1·85
(*i*) $e - \frac{1}{2}f$	1·985	2·445

Mean value of $e \div \frac{1}{2}(a+b) = 0{\cdot}71 \pm 0{\cdot}05$
Mean value of $e \div f = 1{\cdot}88 \pm 0{\cdot}03$

Refractive indices of the media

According to Duke Elder the lens of the human eye is "made of many zones which are not concentric with each other and which vary in optical density very considerably from the periphery to the centre. The most reliable measurements are those of Freytag (1907) who found the index of the cortex at the anterior and posterior poles to be 1·387 and 1·385, while that of the centre was 1·406." The refractive index of the human cornea as determined by Matthiessen (1891) and by Lohnstein (1897) is 1·38. That of the corneal epithelium is higher, being 1·416 (Fischer 1927). Correct to three decimal places the values for the aqueous and vitreous humours of the human eye as determined by numerous investigators are the same, viz. 1·336.

Since the cornea of the eye of *Gasterosteus* is very thin, the relevant data for an approximate reconstruction of the path of the critical ray inside the eyeball are the refractive indices of the vitreous humour and of the lens. Our own measurements of the vitreous humour of *Gasterosteus* determined by means of an Abbé Refractometer were:

1·360	1·370
1·360	1·359
1·360	1·357

This gives a mean value, $\mu_v = 1{\cdot}360$.

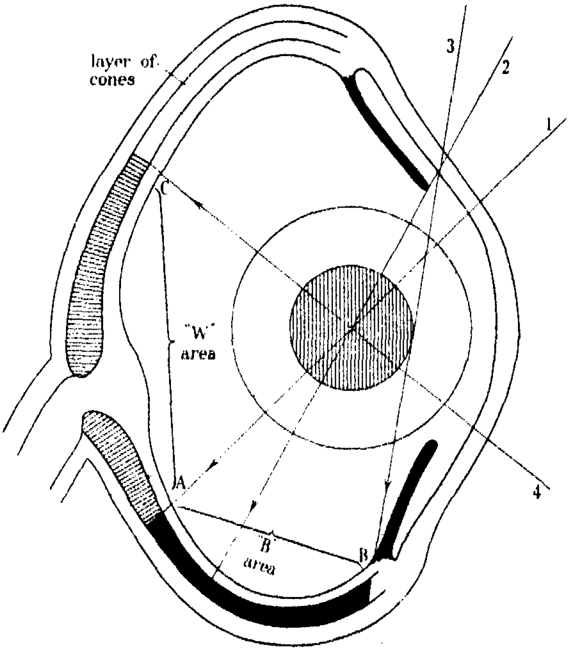

FIGURE 7. The eye of *Gasterosteus*.

The determination of the refractive index of the lens cannot be made with a refractometer. Preliminary attempts to obtain a value by the immersion method (over the range $\mu = 1{\cdot}25$–$1{\cdot}60$) were unsuccessful. For a reason which will appear later this method is unsuitable for the purpose. Two independent methods were subsequently used: (*a*) a comparison of Vernier microscope measurements of the real depth of an object (lycopodium powder) on a glass slide with those of its apparent depth through the vertical optical axis; (*b*) Vernier microscope measurements of the focal distance of the real image of a distant object.

The results obtained in successive determinations by the ratio of the real to the apparent depth differed considerably. The following are typical:

Eye 6	1·41 : 1·39 : 1·40	Mean 1·40
„ 7	1·36 : 1·40 : 1·37	„ 1·375
„ 8	1·40 : 1·46 : 1·44	„ 1·43
	Mean 1·40	

As an independent check on this estimate we next tried the focal distance method. For a spherical lens of focal distance (measured from the centre of the sphere) f radius r and refractive index μ

$$2f(\mu-1)=r\mu.$$

In this formula f and r are easy to measure. The procedure for the lens of *Gasterosteus* is as follows: The lens is removed, placed with a camel hair brush on a piece of filter paper to remove moisture (aqueous humour), and transferred *immediately* to a glass slide on the stage of a Vernier microscope with eye piece micrometer. Images of distant objects reflected by the microscope mirror are found by the lens. Such images are very clear. It is possible to read print or see bubbles from the aerating orifice in an aquarium jar at the end of the room. The relevant measurements of the position of the image and of the upper and lower boundaries of the lens can be made in a few seconds without any danger of desiccation. Values for μ calculated from eight eyes are shown below (table 3). All the fish used were about 7 cm. long.

TABLE 3

	Diam. lens	*Apparent* value of μ	Focal distance
1*a*	1·41	1·365	1·315
2*b*	1·81	1·340	1·695
3*a*	1·58	1·310	1·67
3*b*	1·62	1·33	1·63
4*a*	1·68	1·35	1·62
4*b*	1·67	1·35	1·615
5*a*	1·63	1·32	1·63
5*b*	1·58	1·34	1·58

Mean value of μ = 1·34
Mean diam. lens = 1·62
Mean focal distance = 1·59

The figures for μ estimated in this way are much more consistent than those based on the real-apparent depth method. The mean is very slightly

higher than water. So if they were correct the lens could not function as such when the eye is submerged in water. To test this surprising conclusion we therefore repeated our determination on a glass slide with a high collar containing water into which the microscope objective could dip. We then found that the focal distance of the lens of the eye of *Gasterosteus* is only slightly greater in water than in air. This unforeseen paradox admits of only one explanation which explains the failure of the immersion method and the discrepancy between the means estimated by the real-apparent distance method and from the focal distance in air. The formula used for the latter assumes that the lens is homogeneous. If it is *not* homogeneous the method is not valid.

In stained sections of the eye it is noticeable that the lens has a rind and a concentric spherical core, which is roughly half the diameter of the whole lens. If the outer rind has a refractive index approximately equivalent to that of water it will be optically non-existent when immersed therein, and the inner core alone will be instrumental in forming images. The problem set by the data before us therefore raises the question, what is the refractive index of a spherical lens which has, in water, roughly the same focal distance as a water-lens of twice its dimensions in air? According to the formula the relative refractive index referred to water must be about 1·11 corresponding to an absolute refractive index of roughly 1·5. At this stage we therefore studied the optical properties of a compound spherical lens with an outer shell of water and an inner core of glycerine ($\mu = 1{\cdot}47$) or carbon bisulphide xylol mixtures ($\mu =$ *circa* 1·52).

The properties of these models will be discussed below. Meanwhile it was found that if the lens is immersed in water for about 10 min., it is easy to peel off a relatively soft shell leaving behind an apparently homogeneous core which is extremely resistant to pressure. From seven determinations of the ratio of the radius of this core to that of the whole lens, we obtained the mean value 0·478 with a maximum error 0·027 and a standard deviation of the mean figure equal to 0·004. On this basis the estimated values of the diameter of the core are calculated in the ensuing table (table 4) which gives the focal distance of the whole lens immersed in water, the relative refractive index (μ_1) of the core (referred to water) and its absolute refractive index (μ_2) obtained by multiplying the latter by 1·33. These calculations, which yield a mean refractive index of 1·49 and a focal distance of the lens when immersed of 1·126 times the diameter of the whole lens, are based on the assumption that the outer rind is optically neutral in water, i.e. that its refractive index is approximately the same as that of water.

TABLE 4

	Diam. whole lens	Estimated diam. core	Focal distance	μ_1	μ_2
6*a*	1·80	0·860	2·10	1·11	1·48
6*b*	1·85	0·884	1·944	1·13	1·51
7*a*	1·78	0·851	2·07	1·11	1·48
8*a*	1·81	0·865	2·115	1·11	1·48
8*b*	1·74	0·832	1·97	1·12	1·49
9*a*	1·84	0·879	1·98	1·12	1·49

Mean μ_2 = 1·49
Mean ratio of focal distance to diameter of whole lens = 1·126

Physical models of the lens

The mean focal distance of the whole lens in air is shown in the preceding tables to be $1{\cdot}59 \div 1{\cdot}62$ times the diameter of the latter. Its mean focal distance in water is 1·13 times its diameter. Hence the ratio of the focal distance of the whole lens in air to the focal distance in water is $1{\cdot}59 \div (1{\cdot}62 \times 1{\cdot}13) = 0{\cdot}87$. The radius of the inner spherical core to that of the shell of the lens is approximately 0·475. On the assumption that the air-image is formed by refraction through the shell and the water-image by refraction through the core, the refractive index of the shell is approximately the same as that of water (1·34) and the refractive index of the core is estimated to be about 1·5.

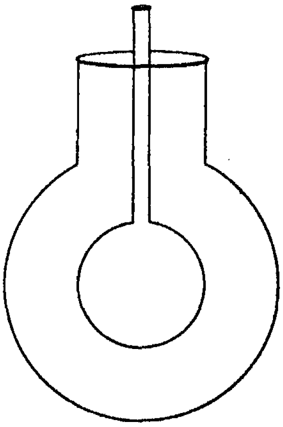

FIGURE 8

The validity of this reasoning depends on whether it is possible to make a model with similar properties. A model which sufficiently reproduces these assumptions may be made by immersing a small spherical flask (figure 8) containing a fluid of high refractive index in water inside a spherical flask with a wide neck. Such combination lenses have the property of forming *separate* images of distant objects in air. The position of the hindmost image approximately corresponds to the focus of a water lens. Since it can be completely eliminated by placing in front of the combination lens a diaphragm with a circular aperture whose diameter does not exceed that of the inner sphere, the hindmost image is evidently formed by refraction through the shell alone.

The measurements made with such models and their relevance to the present topic are subject to limitations arising from the depth of the image and the difficulty of getting flasks of guaranteed sphericity. Hence focal distances calculable from the spherical lens formula are given in parenthesis below for comparison with the observed figures (inches). With a combination of water and glycerine ($\mu = 1{\cdot}47$) the *ratio* of the focal distance of a lens with the dimensions postulated ($c \div a = 0{\cdot}475$) in air to its focal distance in water was 0·75. When a carbon bisulphide and xylol mixture of refractive index 1·51 was substituted the ratio was 0·84. Making allowance for the inaccuracies inherent in the design of the models this is close enough to the mean value 0·87 for the lens of *Gasterosteus* to substantiate the conclusion that the latter consists of an outer shell of approximately the same refractive index as water and an inner core of high refractive index, viz. about 1·5.

TABLE 5

Outer lens (water)		Inner lens		Combination				
Radius (*a*)	Focal distance in air (*b*)	Radius (*c*)	Focal distance in air (*d*)	Inner in air (*e*)	Outer in air (*f*)	In water (*g*)	$c \div a$	$f \div g$
			(*a*) Inner lens of glycerine					
2	3·9 (4·0)	0·95	1·3 (1·47)	2·35	3·5	4·7 (4·75)	0·475	0·75
2	3·95 (4·0)	0·85	1·25 (1·32)	2·25	3·47	4·1 (4·2)	0·425	0·85
2	3·9 (4·0)	0·775	1·175 (1·2)	2·15	3·75	3·7 (3·9)	0·39	1·0
			(*b*) Inner lens of water					
2	3·9 (4·0)	0·95	1·4 (1·425)	2·15	3·2	3·8 (3·8)	0·475	0·84
2	3·95 (4·0)	0·75	1·05 (1·12)	2·0	3·4	3·05 (3·0)	0·375	1·11

Position of the image in situ

Since the refractive index of the vitreous humour is (1·36) very close to that of water, the essential features of the optical system of the eye of the stickleback are reproduced by the lens when it is immersed in water. From sections of fixed material the gap between the lens and the retina appears to vary between about 0·45 in the centre and 0·65 times the lens diameter towards the periphery. Since the focal distance in water is 1·13 (in the same units) or $1{\cdot}13 \div 0{\cdot}50 = 0{\cdot}63$ from the inner surface of the lens, the surface of the peripheral region of the retina corresponds closely to the position where the rays come to a focus. In reality these estimates are biased by distortion due to fixation. The position of the image can be determined directly, if the eye is immersed in water with the lens facing the mirror below the microscope stage. A window can then be cut in the back of the eye, and an image of distant objects formed when the lens is surrounded by the intact cornea on one side and the vitreous humour on the other, can then be seen to be on the inner surface of the latter in the *centre* of the inner wall of the eyeball.

5. THE EFFECTS OF INFERIOR ILLUMINATION

We are now in a position to map the regions of the retina affected when the eye is illuminated from above and below. Under conditions of overhead illumination (figure 7) in a container which does not reflect or scatter light no ray can enter the eye if it is more acute (to the normal) that the critical ray (1) which passes through the optical centre of the inner or effective lens. All rays parallel to the critical ray are brought to a focus on the retina at approximately the same place (A) well below the blind spot. Any ray (2) which is less acute will be refracted outwards towards the periphery. A ray (3) which just fails to penetrate beyond the optically neutral shell of the lens will just reach the outer margin of the retina at B. This means that the region *effectively* concerned with the normal black background response under conditions of overhead illumination lies between the points A and B.

When the fish is illuminated from below in a container which does not reflect or scatter light from the top or sides, the only part of the retina which is illuminated lies between the point C where the critical ray strikes it and the outer margin bounded by the iris. Hence we have before us three initial possibilities to investigate:

(a) If inferior illumination in a black container with a black top reproduces the white background response any part of the retina except the "B" area between A and B may be effectively concerned with the white background response.

(b) If inferior illumination in a black container with a black top evokes the black background response the effective receptor field for the white background response is a central zone defined by a curvilinear boundary with upper and lower limits at C and A, while the effective receptor field of the black background response lies in the entire peripheral zone dorsal to C and ventral to A in vertical section.

(c) If inferior illumination in a black container with a black top evokes the same response as keeping the fish in darkness, the effective receptor field for the white background response is the central zone within the limits C and A, while the peripheral region dorsal to C is *neutral* as far as colour change is concerned.

The arrangement used for comparing the response to inferior and to superior illumination in containers with black sides and black tops or bottoms was the same as that used in other investigations from this laboratory, and has been described already by Smith (1938) in one of its publications. The ensuing table shows that when the container has a black top and black sides, pale or dark sticklebacks *slowly* approach the equilibrium figure characteristic for fish kept in darkness. Both the time relations (table 6) and the equilibrium value to which both sets converge prove that the effect of restricting illumination to the peripheral region dorsal to C is the same as when no light enters the eye.

TABLE 6. INFERIOR ILLUMINATION

Top ...	Black	Black	White	Black
Sides ...	Black	Black	White	White
Initial M.I.	4·7	1·3	4·6	4·7
¼ hr.	4·3	1·8	2·0	2·1
½ hr.	3·5	2·0	1·5	1·7
1 hr.	3·5	2·1	1·3	1·4
4 hr.	3·2	2·3	—	—
24 hr.	2·6	2·4	—	—

This means that the effective receptor field for the black background response is confined within the limits AB and the effective receptor field for the white background response is confined within the limits CA. We may still ask whether the *whole* of the area CA effectively controls the white background response or whether the limits can be made more narrow. The results of comparing the effects of overhead illumination in containers with (*a*) black bottoms and black sides, (*b*) white bottoms and white sides, (*c*) white bottoms and black sides and (*d*) black bottoms and white sides, throw further light on this (table 7). The fish were held in their tubes (figure 2) in the centre of the containers of diameter 5 in. and height 2½ in. Since rays scattered from the floor and from the sides of the container are concurrently necessary to evoke a full white background response we may conclude that the effective W area extends over the whole central zone between the limits C and A above and below the blind spot.

TABLE 7. SUPERIOR ILLUMINATION

Sides ...	Black	White	Black	White
Bottom ...	Black	White	White	Black
Initial M.I.	1·3	4·7	4·6	1·2
¼ hr.	3·5	2·1	3·0	3·0
½ hr.	4·4	1·6	3·0	3·0
1 hr.	4·7	1·4	2·9	3·0

The conclusions stated in the foregoing paragraphs are contrary to those which Butcher (1938) has based on experiments with *Fundulus*. Like ourselves he concluded that the effective *B* area was a ventral region such as that between *B* and *A*. Unlike ourselves he also concluded that the effective receptor field for the white background response is the *entire* region of the retina dorsal to *A*. Butcher's analysis was based on experiments of two types:

(*a*) The effect of rotating the eye *in situ*.

(*b*) Superior and inferior illumination in containers of various types after covering the dorsal or ventral half of each eye with a sheath.

It is possible that the eye of *Fundulus* and the eye of *Gasterosteus* are fundamentally different with respect to their relation to the chromatic function. On the other hand, it is equally possible that the facts described by Butcher admit of an alternative interpretation. Butcher describes his fish as "pale", "intermediate" or "dark", and uses no quantitative rating of the micro-response. He regards "pallor" as diagnostic of the white background response, and gives no indication of how the white background response of *Fundulus* differs from the effect of prolonged exposure to darkness. Concerning the latter, the only recorded information at our disposal is the statement of Parker and Lanchner (1922): "*Fundulus* in the dark is light coloured and on exposure to the light shows a temporary darkening."

These words are consonant with our experience of many species of Teleostei which have been studied in this laboratory. In general teleosts which exhibit comparatively rapid background responses are macroscopically pale in darkness, and may even yield (e.g. trout)* a lower M.I. than for the white background response with overhead illumination. The all-important criterion of the two types of pallor is therefore the *time graph*. Since Butcher does not record information of this kind, the published data concerning the receptive fields in the eye of *Fundulus* are equally consistent with his own interpretation or with the conclusions which emerge from our own study of *Gasterosteus*.

6. THE VISIBLE SPECTRUM OF *GASTEROSTEUS*

The delimitation of two fields in the retina of *Gasterosteus* by the experiments described in the preceding section admits of two possible interpretations:

(*a*) The photo-receptive elements of the *B* and *W* regions are qualitatively different.

---

* Neill (unpublished).

(*b*) While the photo-receptive elements of the *B* and *W* regions are alike, their central connexions are different.

It might be possible to establish (*b*) by the laborious method of nerve degeneration after the destruction of the superficial layers of the retina in one or other of the regions defined. Alternatively, (*a*) could be established if there were a visible histological differentiation of the local photo-receptors or if the response of the latter could be shown to depend on circumstances other than the accident of situation. Hogben and Slome (1936) have recorded a qualitative difference of the *B* and *W* receptors in the eye of *Xenopus*. In this species their experiments indicated that the black and white background response are sensitive to different regions of the spectrum.

Few recorded observations on the relation of wave-length to chromatic behaviour are conclusive. The literature of the subject contains many records of response to coloured background without any information to indicate the absorption spectrum of the pigments used. So the assertion that a particular type of response occurs when an animal is placed on a red background might for example signify the direct effect of rays in the red region or the result of reducing radiation in the green region. The reflection data for two red and two yellow pigments as given below (table 8) are worth citing to emphasize the erroneous conclusions which can be drawn from observations of this type.

TABLE 8. RELATIVE REFLECTION BY PIGMENTS

	Wave-length in mμ							
Pigment	440	480	520	560	600	640	680	700
Tuscan red	29	29	33	33	66	83	96	100
American vermilion	12	8	10	14	37	81	100	100
Yellow ochre	31	32	49	80	100	91	90	90
Chrome yellow (medium)	6	7	22	80	97	100	100	100

A less elementary ambiguity is a common feature of many observations by investigators who have taken the precaution to study the effect of monochromatic light transmitted by standard filters without regard to the total energy transmitted or to variations of the total energy emitted by the source in different regions of the spectrum. To discriminate between the specific effect of the region selected and the total energy content of the beam transmitted we may adopt one of two methods. If the total energy in different regions of the spectrum emitted by the source and the percentage absorption curve of the filter are both available, we can assign values of total energy transmitted through a given filter, and compare the reactions of animals exposed to different levels with different filters. This was the method used by Hogben and Slome (1936) in work on *Xenopus*. Alternatively, we may arrange the distance of the source to transmit equivalent energy of radiation when one filter is substituted for another by direct measurement with a photronic cell whose sensitivity for equivalent energy absorbed is known. This is the method which we have used in the present investigation.

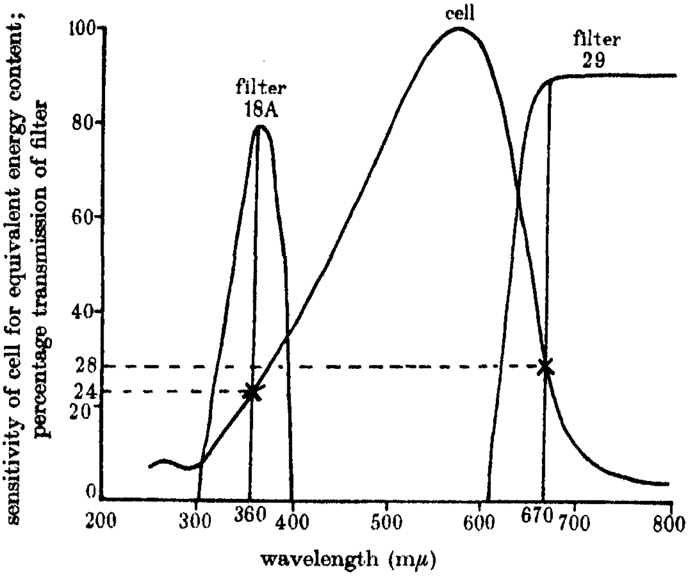

FIGURE 9

The procedure is indicated in figure 9. The middle curve ("cell") shows the relative sensitivity of the Weston photronic cell for equivalent energy content in different regions of the spectrum. The two curves for the filters give the percentage transmission at various wave-lengths. The point where the mean ordinate of the filter-transmission curve cuts the cell-sensitivity graph enables us to read off the sensitivity of the cell for the same energy content at the mean wave-length transmitted by the filter. Thus if the cell reading for a *fixed* position of the source is X μA when filter 18 A is used, the source must be raised or lowered till the cell delivers $(24 \div 28)\, X$ μA if we wish to get a beam of equal energy content from the same source on substituting filter 29 for 18 A.

The sense in which the "mean ordinate" of filter 29 is here used needs to be specified. All the filters used transmit freely in the infra-red, i.e. > 700 μμ. Hence it is not possible to give a mean ordinate for each filter unless we confine ourselves to wave-lengths $< 700\ \mu\mu$. Since experiment showed that *Gasterosteus* gives no reaction to radiation above $700\ \mu\mu$, we are entitled to regard the region of the spectrum beyond this limit as neutral for the object of the present enquiry. The source of illumination was a *Hanovia* mercury lamp, enclosed in light-proof box with cooling device and window to hold the filter or combination of filters used. It was suspended by a pulley from the roof so that it could be lowered or raised to give the appropriate reading of the galvanometer in circuit with a photronic cell. The following table gives the distances of the same source at which we obtained a cell current corresponding to equal energy content transmitted by the Wratten filters specified.

TABLE 9

Filter	Range in $\mu\mu$	Mean wave-length	Sensitivity of cell at mean wave-lengths	Galvo. divisions	μA	Distance in in.
18 A	300–400	360	24	8	0·20	150
45 + 48	420–520	460	60	20	0·50	7¼
15 + 58	510–620	540	94	31	0·77	40
29	600–	670 (600–700)	28	9	0·22	30
88	700–	750 (700–800)	5	2	0·05	14

The time graphs given in the preceding section were based on illumination with different sources, namely daylight, or a tungsten lamp. When the tungsten source was placed to give "dim" illumination in these experiments the photronic cell in the position occupied by the container for the fish yielded a current of 0·63 μA. The mean wave-length for the tungsten lamp is about $580\ \mu\mu$ in the range 400–700, and at this value the relative sensitivity of the cell is 100. For the same energy content as transmitted by filter 18 A in table 9 the cell current would be $100 \div 24(0{\cdot}20) = 0{\cdot}83$ amp. Hence the intensity of the dim tungsten light in the foregoing experiments was less than that transmitted in the experiments below, and since the fish responded to dim tungsten illumination any differences which could manifest themselves when they were illuminated with filtered monochromatic light, could not be due to intensity *per se*.

The next table (table 10) embodies the results of observations on the effect of exposing fish for the periods stated to filtered light standardized for equivalent intensity as indicated in table 10 by varying the distance of the source. The data show: (*a*) that there is no detectable difference between the sensitivity of the "*B*" and "*W*" areas of the retina; and (*b*) that the limits for both are approximately the same as for the human eye, i.e. the visible spectrum of *Gasterosteus* does not extend significantly beyond the limits 400–700.

TABLE 10. DARKNESS TO MONOCHROMATIC LIGHT

Filter	Colour		Av. M.I. after 10 min.	Av. M.I. after 20 min.
18 A	U.V.	*W*,	2·1 (6)	2·0 (6)
		B,	2·2 (6)	2·2 (6)
45 + 48	Blue	*W*,	1·7 (12)	1·4 (6)
		B,	4·3 (6)	4·7 (6)
29	Red	*W*,	1·5 (6)	1·3 (6)
		B,	4·3 (6)	4·7 (6)
88	Infra-red	*W*,	1·9 (6)	1·8 (6)
		B,	2·2 (6)	2·3 (6)

7. MICROSCOPIC STRUCTURE OF THE RETINA

In the absence of evidence pointing to a qualitative difference between the responses of the *B* and *W* areas to monochromatic light, histological examination of the retina might still indicate structural differences. We have not yet been able to establish any certain correlation of this kind. The only positive indication which is relevant to the experimental data is the behaviour of the retinal pigment cells. In darkness the latter are contracted towards the choroid and the photoreceptive elements extend outwards in the same direction. In the eye of sticklebacks kept on a white background with overhead illumination, the pigment cells are uniformly expanded, i.e. the pigment is dispersed throughout the cell processes which arborize between the photoreceptive elements, and concentrated at their extremities just below the base of the photoreceptive elements. The latter appear to be somewhat shortened; but such photo-mechanical movement is relatively slight. The condition of the pigment in the eye of a stickleback exposed for a suitable time to overhead illumination on a black background is not uniform. Throughout the *B* area of figure 7 the condition of the pigment and the photoreceptive elements is as in the eye of a stickleback subjected to overhead illumination on a white background. Elsewhere the pigment is dispersed along the branches between the photoreceptors, but is not concentrated at their extremities. To this extent the behaviour of the pigment cells confirms the analysis of § 5.

Among the photoreceptive elements of the eye of *Gasterosteus* we have not been able to draw a clear distinction corresponding to the rods and cones of Tetrapoda. Single and twin "cones" occur in both *B* and *W* regions. The distance between the choroid and the basement membrane of the *B* area is shorter than the corresponding distance in the *W* area. This is associated with the fact that the cones of both sorts appear to be shorter in the *B* area.

8. IMAGE FORMATION IN THE EYE OF THE STICKLEBACK

The foregoing analysis of the optical properties of the lens of the eye of *Gasterosteus* furnishes new information about the localization of images. For reasons explained in § 5 the limits of refraction are roughly the points *P* and *R* (figure 10) where the ray which grazes the iris and passes through the centre of the lens strikes the retina. Image formation is therefore restricted to the area defined by *P* and *R* and the entire region peripheral to this can only register light and shadow. Within the region *PR* in figure 10 we can distinguish a ventral segment defined by *R* and by *Q* where the critical ray through the centre of the lens strikes the retina. All images of terrestrial, i.e. extra-aquatic, objects are restricted to this segment.

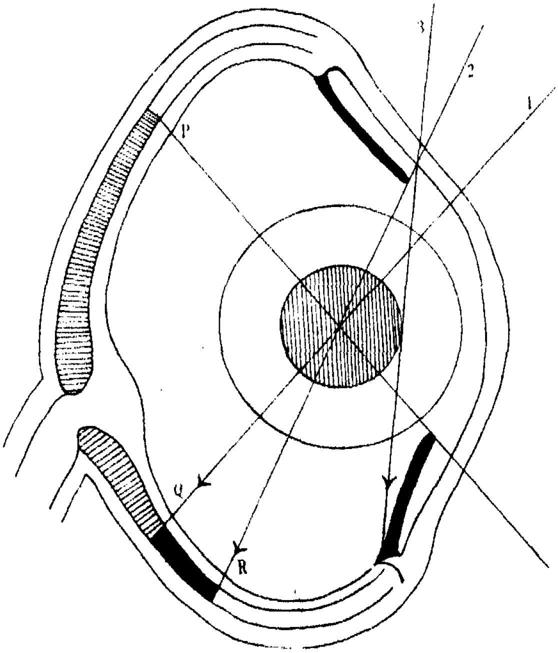

FIGURE 10

From the standpoint of image formation we may therefore distinguish the following regions of the retina:

(*a*) A small segment *QR* below the blind spot where images of terrestrial objects are formed.

(*b*) A central zone *PQ* for images of subaquatic objects including the bottom of the vessel, pond or river.

(*c*) A peripheral zone where no clear images are formed.

The "*B*" area is made up of (*a*) and *possibly* also of the ventral part of (*c*). The "*W*" area corresponds to (*b*). The possibility of forming simultaneous images of one and the same object in both eyes is excluded by the position of the eyes. Stereoscopic vision is impossible.

It is natural to ask how far this picture of retinal localization is characteristic of fishes as a group. A few observations made on the lens of *Raia clavata* indicated that its optical properties are substantially the same as those of the lens of *Gasterosteus*. Owing to the presence of the characteristic dorsal membrane which lies inside the conjunctiva of their eyes, it seems that skates form no images of the extra-aquatic world. With one exception the lenses of species from widely divergent families of fishes which we have cursorily examined had the same structural dimensions as that of the stickleback, consisting of a relatively small, hard, highly refractive core and a thick outer shell. Through the kindness of Dr G. L. Purser we have been able to examine sections of the eye of *Calamoichthyes*. The lens of *Calamoichthyes*, like the lenses of other fish, consists of a spherical core and a concentric shell, but the latter constitutes a relatively thin pellicle around the central zone, which makes up the bulk of the lens.

Owing to the great hardness of the core of the lens and the much greater distensibility of the peripheral shell, a muscular ciliary body could have no effect on the refractive properties of a lens such as that of *Gasterosteus* or other fishes which we have examined, and would therefore have no selective value. In the evolution of vertebrates the transition from the aquatic to the land habit seems to have been accompanied by the appearance of a new type of lens structure as a necessary preliminary to the tetrapod mechanism of accommodation.

SUMMARY

Chromatic behaviour of *Gasterosteus* is controlled by a humoral and by a nervous mechanism. The former alone plays any significant part in the transition to the equilibrium condition in darkness. It reinforces nervous control in the transition to the equilibrium condition during background reversal with overhead illumination.

The photoreceptors concerned with the black background response are located in the floor of the retina below the optic nerve, and the photoreceptors concerned with the white background response are located in a restricted region in the centre of the retina above and below the optic nerve. With respect to colour change the dorsal region of the retina is neutral.

The lens of the eye of *Gasterosteus* consists of an outer spherical shell and a concentric spherical core. The refractive index of the former, like that of the vitreous humour, does not differ significantly from that of water. The core which has a high refractive index in the neighbourhood of 1·5 is the effective refractive constituent of the optical system.

In relation to image formation, we may distinguish three regions of the retina: (*a*) below the optic nerve, a small area where images of extra-aqueous objects are focussed; (*b*) around the optic nerve, a larger region where images of subaqueous objects are formed; (*c*) a wide band of the periphery where only light and shadow are registered.

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The chromatic behaviour of the eel (*Anguilla vulgaris* L.)

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1. Introduction

Neill (1939) has described the chromatic response of normal and blinded eels to various conditions of illumination. From the times taken to equilibrate when passing from white to black "background" and vice versa with overhead illumination, he concluded that co-ordination is humoral. The time taken to equilibrate after transference to darkness from an illuminated white background or vice versa implies that control is bihumoral.

Apart from Neill's work and an early comment by Petersen the chromatic behaviour of the eel has engaged little attention. Lode (1890) described contraction of the melanophores after faradic stimulation of the cord. Odiorne (1933) found that injection of posterior lobe pituitary extract caused expansion of the melanophores. The present communication deals with experiments designed to elucidate the mechanism of co-ordination more fully. It includes observations on (*a*) modification of normal chromatic behaviour by total or partial hypophysectomy; (*b*) effect of total and partial hypophysectomy on tolerance to pituitary extracts; (*c*) influence of nervous stimulation in the presence or absence of an intact blood supply. They are based chiefly on the behaviour of the dermal melanophores for recording which the melanophore index (μ) of Hogben and Slome (1931) is used throughout.

2. The pituitary gland of the eel

For the elucidation of the experiments to be described in what follows, it is first necessary to indicate the morphological peculiarities of the pituitary of the eel and the means adopted for removing it from live fish. The pituitary of the eel is divisible histologically into four parts (fig. 1):

(1) An anterior lip of epithelial cells surrounding well-marked vesicles (= anterior lobe of de Beer 1926).

(2) The main mass of the anterior lobe composed of closely packed glandular cells (= *Übergangsteil* of de Beer).

(3) The *pars nervosa* of fibres continuous with those of the third ventricle.

(4) A glandular *pars intermedia* surrounding (3).

De Beer's interpretation of the homologies of these parts in teleosts followed the earlier accounts of Stendel (1914), and are based on the cod. In its general characteristics the pituitary of the eel recalls de Beer's description of *Amia* from which it differs more especially in so far as it does not retain its connexion with the pharynx. There is also no doubt about the essential similarity between the pituitary of the eel and that of the cod as de Beer describes it. For this reason the present writer believes that de Beer's *Übergangsteil* is the anterior lobe *sensuo stricto*, and that the anterior lip which de Beer identifies as the pars anterior is really equivalent to the unseparated pars tuberalis of the Urodele. According to this view the ganoid pituitary takes its proper phyletic position between that of the teleost and that of the Urodele.

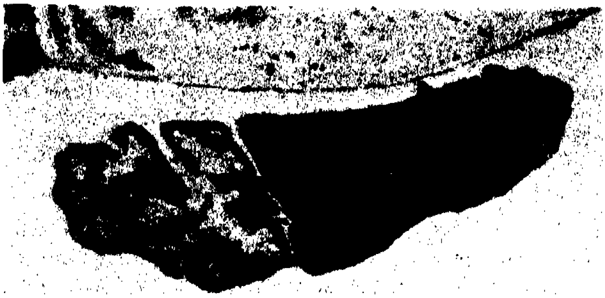

FIGURE 1. Longitudinal section of the pituitary.

Longitudinal sections of the eel pituitary sometimes display a narrow cleft between regions (1 and 2) and (3 and 4). It is rarely so obvious as in the one illustrated. Macroscopically the cleft is not apparent but regions (1 and 2) are distinguishable by shade, consistency and position from (3 and 4).

Hypophysectomy of the eel can be accomplished by the method described by Hogben (1923) for the frog. The eel is anaesthetized by immersion in urethane solution. Anaethesia is complete and prolonged. If wrapped in a damp duster and laid on the bench, the animal requires no attachments. The gap is kept open by forceps, a piece of card being inserted over the tongue. The mucous membrane is deflected down the middle line. Approximately ¼ in. internal to the angles of the jaw two branches of the carotid pass into the skull and unite on the surface of the anterior lobe. The bony floor of the skull above the pituitary is *lightly* drilled with a dental burr. When sufficiently thin, it is divided with a scalpel down the middle line and two small flaps pulled laterally. The frog has a comparatively thick chrondocranium. In the eel there is only a tough membrane. This is slit medially, leaving the pituitary in an accessible position with the two intact carotids lying on its surface. Removal of the whole pituitary can then be done simply by applying suction. Removal of either lobe separately is difficult. Several methods have been tried. The best is to apply light suction to the lobe to be removed and then to snip it free from the portion still in situ. Alternatively the anterior lobe can be destroyed with a cautery. Some bleeding is inevitable whenever the anterior lobe is removed. It is imperative to leave the carotid union undisturbed until suction is applied, otherwise the restricted site makes it quite impossible to be certain about what part is removed.

3. EFFECT OF PREVIOUS CONDITION ON EQUILIBRATION AFTER HYPOPHYSECTOMY

For the experiments described below, both eels from which the whole pituitary gland was removed, and eels from which the anterior lobe only (i.e. regions 1 and 2) was removed, were used. The bulk of the operative stock, consisting of about forty to fifty *totally* and twelve *partially* hypophysectomized eels, were kept in the laboratory under continuous observation for a period of 1–6 months. Post-operative mortality was negligible. The eels on which the operation had been performed were not less viable than normal eels kept under the same laboratory conditions.

During transition from one illuminated background to another, and on reaching equilibrium after hypophysectomy, all the dermal melanophores of the body do not react simultaneously. This is particularly noticeable after hypophysectomy, when melanophores near to the spinal cord equilibrate at a higher figure than do those at the edge of the fin. There is, in fact, a well-marked gradient. The practice adopted in the ensuing experiments was to take readings at four easily identifiable points on the tail of each fish. The points on the graphs reproduced below represent the average figure from these four sets of observations.

When the *whole pituitary* is removed from eels with expanded melanophores, they pale slowly, and gradually settle down to an intermediate colour. The average melanophore reading from a large number of animals subjected to continuous bright illumination in a black tank is 3·3, at 10–15° C. In black tanks with dim overhead illumination of exactly the same intensity as is found favourable for eliciting maximal pallor in normal animals on white grounds, the average melanophore reading is 2·7. This shows that the primary response is independent of the activity of the pituitary, as in *Xenopus*. Removal of only the *anterior lobe*, including the lip (1), results in expansion of the melanophores under all conditions of illumination. In the majority of such animals expansion is maximal in a white tank, but in two maintained in these conditions for 9 months the index was lowered to approximately 4. The reason for this is being investigated in a long term experiment which will be reported on later.

In early experiments it was noticed that some animals were paler two days after total hypophysectomy than subsequently. Since Neill's observations on the natural response suggest that a contracting hormone, *W*, is *more slowly* excreted (or destroyed) than an expanding hormone *B*, a reasonable interpretation of this finding is that *W* remains in the circulation for a longer period than *B* after the removal of the source of both of these hormones, and that both are, in fact, located in the pituitary gland.

In my experiments this hypothesis was first tested in the following way. Two groups of animals were taken.

Group *a* had been kept for 6 months previously in illuminated black tanks. Group *b* were placed in a white tank until the mean melanophore index was 4·2. The animals were then completely hypophysectomized and placed in an illuminated black tank. Their subsequent responses are shown in the accompanying graph (figure 2).

If the behaviour of the two groups is due to the fluctuation of a single (expanding) hormone *B*, the only difference between them is that *b* had less *B* hormone in the circulation than *a*. If so, we should expect that the melanophore index of the second group would diminish consistently to a common position of equilibrium. We might also expect that it would fall more gradually in its initial stage than does the melanophore index of the other group. The fact that it actually falls more steeply, drops well below the equilibrium condition to which it then gradually rises, is easily explainable if we assume that the initial difference between *a* and *b* is the presence in the latter of a second hormone, *W*. On this assumption, the *W* content of the circulation in the first group would be minimal under conditions of overhead illumination in a black tank. On the other hand, the *W* content of the blood in the second group would be appreciably higher, and since Neill's observations suggest that *W* is eliminated much more slowly than *B*, if this hypothesis is correct the sudden drop below the common equilibrium value shown by the second group would be the natural consequence of rapid removal of *B*, and the relatively slow removal of the supra-minimal quantity of *W* in the circulation.

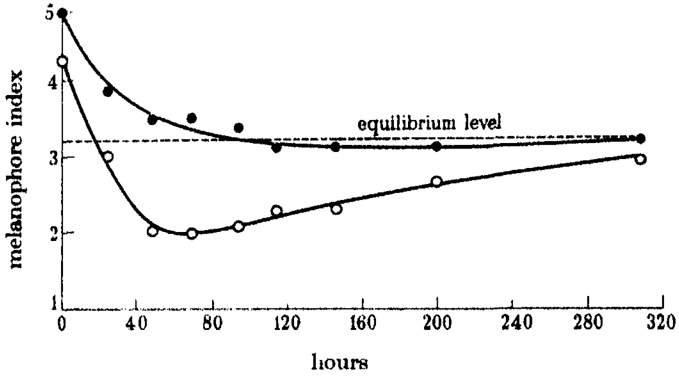

FIGURE 2. Reaction of two groups of eels after total hypophysectomy. ● = Group *a* previously equilibrated at $\mu = 5$. ○ = Group *b* previously equilibrated at $\mu = 4{\cdot}2$. Illuminated black tank.

4. EFFECT OF HYPOPHYSECTOMY ON TOLERANCE TO PITUITARY EXTRACTS*

The hypothesis advanced in the preceding section can be tested in another way. Hogben and Slome (1931, 1936) produced evidence for the existence of *W* in *Xenopus* by injecting *B*-containing extracts into totally hypophysectomized, partially hypophysectomized, and intact (pale) toads. The results showed that the presence of the anterior lobe (with the associated pars tuberalis) is antagonistic to the action of *B*, which is secreted by the pars intermedia. Waring (1938) used a similar method in experiments upon elasmobranchs. The same method has given comparable results when applied to the eel. Equal quantities of posterior lobe pituitary extract, freed from pressor properties by treatment with cold caustic solution (Hogben and Gordon 1930), were injected into normal and totally hypophysectomized eels of equal weight. The eels used had been kept in a white tank. Those of the second group had been operated on 6 months previously. The responses of the two classes of animals is shown graphically (figure 3).

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* This section received in revised form 30 October 1939.

It has been pointed out previously that such results are inexplicable except on the assumption that the presence of the anterior lobe antagonizes the injected *B* hormone. After removal of the whole pituitary gland, the *B* content in the circulation must be lower, so if the *B* hormone of the pars intermedia is the only contribution that the pituitary makes to co-ordination of chromatic control in the eel, we must assume that eels which have no pituitary should be more tolerant on that account. The graph shows that in fact they respond more readily to *B*-containing extracts. The most reasonable explanation of this is that the pituitary of the normal animal is capable of secreting something which antagonizes *B*. If a smaller dose of pituitary is injected the difference between the two groups with respect to the speed of the melanophore expansion is greater (figure 4).

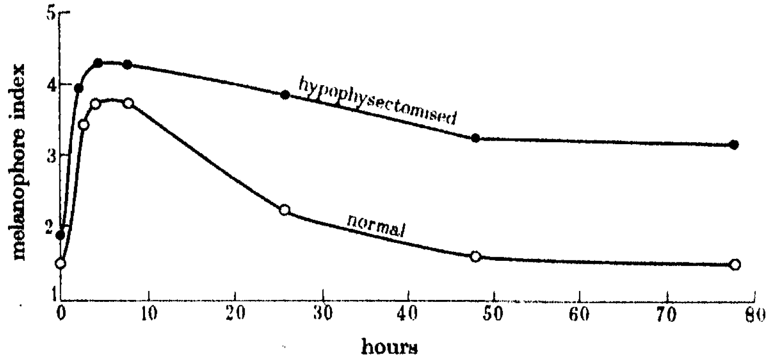

FIGURE 3. Responses of normal and hypophysectomized eels of equal weight to equal injections of posterior lobe pituitary extract freed from pressor properties by treatment with caustic soda. White tank.

A comparison of the graphs in figures 3 and 4 reveals a striking difference in the behaviour of the normal eels. They respond to the larger dose by attaining maximum response ($\mu = 3{\cdot}8$) within 10 hr. and regaining their previous state ($\mu = 1{\cdot}5$) within 50 hr. after a steep initial drop during which the M.I. has fallen to 2·2 25 hr. after injection. There is a corresponding difference between the behaviour of the hypophysectomized groups though the difference is less pronounced. A significant feature common to both groups is that *the quantity of B in excess of what is sufficient to raise the* M.I. *to approximately* 3·5, *i.e. its equilibrium value in darkness, is eliminated more rapidly than the quantity of B necessary to bring the μ up to the same level*. Two general hypotheses might be invoked to explain this difference. On the one hand a direct action of the hormone on tissue metabolism, or on the excretory function of the kidney at a certain threshold might lead to more rapid destruction or excretion of B. On the other hand, we may be dealing with a common characteristic of diffusion gradients. There is at present no way of deciding conclusively that the former alternative is wrong, but it may be pointed out that whether we adopt a *bihumoral*, or could accept a *unihumoral*, hypothesis, the analysis of the normal time-graphs for background equilibrium and transition from equilibrium in conditions of illumination to darkness and vice versa points to the conclusion that the elimination of one or both hormones is more rapid in excess.

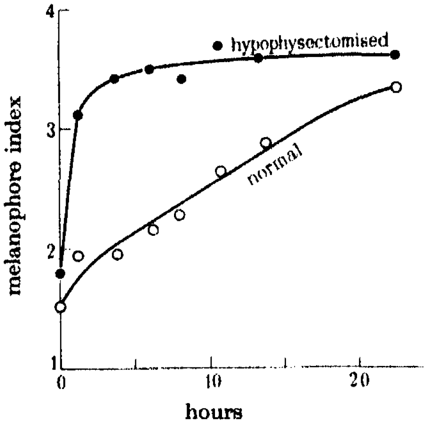

FIGURE 4. Responses of normal and hypophysectomized eels of equal weight to equal injections of posterior lobe pituitary extract freed from pressor properties by treatment with caustic soda. Smaller dose of pituitary extract than used in figure 3. White tank.

The outstanding features of the data summarized in the graphs in figures 3 and 4 is that the response of the hypophysectomized animals develops more rapidly and subsides more slowly than that of the normal ones to the same dosage. This receives a straightforward explanation if we assume that the circulation of the normal animal contains an antagonistic hormone W competing with B. On this assumption more B will be necessary to maintain the threshold ratio of B and W concentrations to a different value of μ, hence tissue destruction or excretion of smaller quantities of B will result in a larger drop in the value of μ. Since the nerve supply is intact in both groups of animals, we need not consider the threshold value of B necessary for the tonic influence of the peripheral nerve supply described below as a relevant circumstance in this context.

5. PHOTIC RESPONSE OF HYPOPHYSECTOMIZED EELS

Unlike other animals (Amphibia, elasmobranchs) which have a slow background response based on bihumoral pituitary control, totally hypophysectomized eels show a limited reaction to illuminated backgrounds. In dimly illuminated black tanks they settle down to $\mu = 2{\cdot}7$. In white tanks with equal overhead illumination they equilibrate at 1·8. This reaction is not shown by blinded hypophysectomized animals. It is comparatively fast. Six animals hypophysectomized a month previously and maintained in a white tank for 5 days had an average dermal index of 1·8. By reading individuals at 10 min. intervals to ascertain the progress of the change, it was found that after 30 min. the average of the six was 2·7. This is exactly the same average figure that the same six animals attained previously after a 2 weeks' sojourn under similar conditions. The reverse change was ascertained in the same way. It took 45 min. for an average index of 2·7 to become 2·0. This is clearly a visual response which can only be brought about by:

(*a*) Vaso-motor changes.

(*b*) Secretion of adrenalin or some other hormone which is *not* secreted by the pituitary gland.

(*c*) Direct innervation of the melanophores.

6. THE ROLE OF PERIPHERAL INNERVATION

The following observations were made in the course of experiments undertaken to discriminate between these possibilities:

(1) Faradic stimulation of the palate or the cord of *normal black-adapted animals* results in contraction of the melanophores. Contraction begins about 3 min. after the start of stimulation and proceeds fairly rapidly so that contraction is complete in about 10 min. They expand rapidly when stimulation is stopped.

(2) Reversible contraction is also evoked when *completely hypophysectomized* animals are used. In these, however, stimulation must be continued for about 15 min. before appreciable movement takes place. The animals used had all been operated on at least a month previously.

(3) Hearts were removed from normal black-adapted animals. The specimens were left for 10 min. during which time all circulation stopped. Faradic stimulation again resulted in contraction but only after 15–20 min. Expansion rapidly followed cessation of the stimulus and contraction could be brought about by further stimulation.

(4) Exactly the same procedure was adopted as in (3) above, except that completely hypophysectomized fish were used. Contraction started in 10–15 min.

The more significant data from these experiments can be tabulated thus:

(i) Stimulation of the palate region or cord elicits pallor in normal or hypophysectomized eels even after the circulation has been stopped.

(ii) All the changes described are completely reversible.

(iii) There is a significant difference in the reaction time between the groups specified above.

To assess these results correctly we have to take into account two other known facts about the pituitary gland. The first is that vaso-motor tone, at least in land vertebrates, is maintained by the pressor constituent of posterior lobe extracts and that the pressor substance is known to be present in the pituitary gland of teleosts. Thus, taking all the relevant data into consideration, the following conclusions may be drawn from the foregoing observations:

(i) That there is direct innervation of the melanophores.

(ii) That stimulation in normal animals causes contraction by vaso-constriction *together with* stimulation of the melanophore nerves.

(iii) Vaso-constriction favours melanophore contraction by *reduction* of the available quantity of the pituitary hormone *B*, and not by the reduced supply of other blood constituents.

(iv) They furnish no evidence for the secretion of adrenalin.

7. DISCUSSION

The chromatic behaviour of cyclostomes, Amphibia and Crustacea is distinguished by slow response and humoral co-ordination. Teleosts such as *Lebistes* or *Gasterosteus* have more rapid responses to change of visual field which is consistent with the predominance of direct nervous control, but their responses in passing from illuminated tanks to darkness and the reverse are comparable to those of the first group. In contradistinction to the various *ad hoc* hypotheses advocated by Parker and his colleagues, Hogben and Landgrebe (1939) have put forward a generalized theory which embraces all these facts. It is that in all forms the melanophore expansion in passing from *darkness* to illuminated black grounds or the reverse is due to the build-up or excretion of *B*. The transition from illuminated black to white grounds or the reverse is due to the build-up or excretion of *W* in Amphibia and elasmobranchs. In *Salmo* and *Lebistes*, in which the background response is extremely rapid, the *W* mechanism has been largely superseded by direct nervous control. To a lesser extent the same is true of *Gasterosteus*. The eel is the first species which provides evidence for the co-existence of a mechanism of bihumoral co-ordination and a mechanism of direct nervous control of visually determined chromatic behaviour. It also provides the only available evidence for the existence of a mechanism of direct nervous control which does not as yet exert any appreciable influence in modifying the dominant and probably more archaic humoral co-ordination in the *intact* fish. From an evolutionary standpoint, the view that recorded differences of response to injection of hormones, removal of glands and stimulation or section of nerves are due to relative dominance of one or other constituent of a co-ordinating apparatus which has three separate components is intrinsically more likely than the various *ad hoc* hypotheses which have been advocated by Parker and his co-workers.

The expenses of this investigation were in part defrayed by a grant from the Carnegie Fund for the Scottish Universities.

I am indebted to Professor Hogben for criticism and advice.

8. SUMMARY

1. There is a dominant bihumoral control of the dermal melanophores of the eel.

2. The expanding hormone *B* is localized in the posterior lobe of the pituitary.

3. The contracting hormone *W* is localized in the anterior half of the pituitary.

4. *B* is built up quickly and excreted quickly.

5. *W* is built up slowly and excreted slowly.

6. There is direct innervation of the melanophores. The time relations of the natural responses show that it has no significance in the behaviour of normal animals. It can only be distinguished in the absence of *B*. Release from nervous control might be expected to show itself in the transition from a white illuminated background to darkness, but the normal behaviour is dominated by the slow excretion of *W*.

7. The results of this investigation support the generalized hypothesis of chromatic co-ordination outlined by Hogben and Landgrebe.

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Cytological studies in *Oenothera* with special reference to the relation of chromosomes to nucleoli

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INTRODUCTION

Compared to the vast amount of work done on the cytology of this group of plants very little attention has been paid to a systematic analysis of the somatic chromosomes. The necessity of such a study was felt long ago by de Vries and Boedijn (1923, 1924), who tried to analyse the chromosomes of *Oenothera Lamarckiana* and its several mutants. Since then the somatic chromosomes of *Oenothera* species have been illustrated from time to time but detailed observations in this connexion remained to be made. Such a situation has probably arisen from the fact that geneticists realized that information from such a study would not throw much light on elucidation of their genetical observations, as all the species of *Oenothera* showed a stable chromosome number of $2n = 14$, without any significant variation in their morphology. Probably a more important factor explaining the dearth of literature on this subject is the technical difficulty involved in getting good cytological preparations of root-tips of *Oenothera* species.

A new line of research has been opened up since the relation of Sat-chromosomes to nucleoli was discovered by Heitz (1931*a*,*b*) and McClintock (1931, 1934). The application of their theory regarding the correspondence of the number of nucleoli to the number of Sat-chromosomes is throwing considerable light on the understanding of relationship and probable origin of some of the different groups of plants. In view of these facts, a study of the somatic chromosomes of *Oenothera* species in relation to nucleoli was thought extremely desirable. Considerable difficulty has been overcome by the recent discovery of a method described by Semmens and Bhaduri (1939) and Bhaduri (1938), by which the chromosomes and the nucleoli can be stained differentially. The present study is part of a contemplated exhaustive study of the chromosomes in different species of *Oenothera* as well as other related genera.

While studying the catenation of a number of species and hybrids of *Oenothera*, during the summer of 1938, an attempt was also made to correlate the relations of chromosomes with nucleoli during meiosis as well as during mitosis.

MATERIAL AND METHODS

For a study of somatic chromosomes root-tips were collected from the following species* and hybrids of *Oenothera* which were grown in the greenhouse at the Courtauld Genetical Laboratory, Regent's Park:

(1) *O. Hazelae* Gates var. *parviflora* Gates.
(2) *O. ammophiloides* Gates and Catcheside.
(3) *O. angustissima* Gates var. *quebecensis* Gates.
(4) *Oenothera* sp. undescribed, from St Jerome, Quebec.
(5) *O. biformiflora* Gates var. *cruciata* Gates.
(6) *Oenothera* sp. undescribed, from Moose Jaw, Sask.
(7) *O. Lamarckiana* de Vries.
(8) *O. blandina* de Vries.
(9) *O. Hookeri* Torrey and Gray.
(10) *O. biformiflora* var. *cruciata* × *O. Lamarckiana*.
(11) *O. missouriensis* Sims.†

Root-tips were also collected from seeds, which were germinated on moist cotton-wool in petri dishes and kept near the radiator in the laboratory at King's College. It was found extremely difficult to obtain satisfactory fixation of the chromosomes when the root-tips were collected from germinating seeds. On the other hand, the freshly developed adventitious roots from the hypocotyl of plants grown in sand gave quite satisfactory fixation with Levitsky's fixative (chromic acid 1 % and formalin 10 % in proportion of 1 : 1). It is well known that fixation images of plant cells vary according to conditions of the tissue at the time of fixing; this was especially apparent in the case of *Oenothera* roots. Levitsky (1931) mentioned that roots collected from germinating seeds grown at low temperatures, such as 15–16° C, showed chromosomes much shorter and thicker, without revealing constrictions, than when they were grown at higher temperatures such as 32° C. Even the frequency of division figures was found to be much less in the former conditions. This observation has been corroborated and it was found further that best fixation can be obtained if the plants are grown in sand and watered once, 2–3 hr. before fixation.

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* Most of the new species and varieties studied in this paper were described by Gates (1936).

† This is a hybrid of two different strains; one from western North Dakota, with pink spots on the sepals, the other of unknown origin but without pink spots (see Gates 1939).

Paraffin sections were made, 8–14μ thick, and the preparations were stained with Feulgen-light green following the method given by Semmens and Bhaduri (1939) and Bhaduri (1938), as well as with Newton's gentian violet iodine method. To obtain clearer preparations and better definition of the microscope image it was found necessary to replace Canada balsam (which is generally acidic) by another medium which is called "Sira" mountant and is prepared by Messrs Stafford Allen and Sons, Ltd., London. The optimum time of hydrolysis of sections for Feulgen staining was found to be 20 min.

For the study of catenation, observations were chiefly made from smear preparations, following the method given by Catcheside (1935). In addition, flower buds were also fixed in Medium Flemming after a pretreatment in Carnoy's fluid and subsequent washing in water.

The observations and drawings were made using a Zeiss 2 mm. apochromatic objective 1·4 N.A. with achromatic aplanatic condenser 1·3 N.A., homogeneous immersion and compensating eyepieces × 20 and × 15.

PREVIOUS WORK ON THE SOMATIC CHROMOSOMES OF *OENOTHERA*

The study of the somatic chromosomes of *Oenothera* began with the observations of Geerts (1907) on *O. Lamarckiana*. He observed differences in the lengths of the somatic chromosomes. Levitsky (1931) later pointed out that "how far these differences are determined by actual chromosome dimensions and how far by the position of the latter with regard to the optic plane remains unknown". The next observations were made by

Gates (1912) in a critical study of the nuclear changes during the whole mitotic cycle of *O. lata*, a mutant of *O. Lamarckiana* with $2n = 15$ chromosomes. He suggested that the *lata* characters are probably constantly associated with the presence of the extra chromosome. He also found some stages showing a case of "somatic reduction". Lutz (1916), after examining the chromosomes of *O. Lamarckiana* and some of its mutations, came to the conclusion that the somatic chromosomes do not show any difference in dimensions and shape. She also found a small additional chromosome fragment in some of the mutants. Davis (1909) made a general study of the behaviour of chromosomes during first premeiotic mitosis in the archesporial cells of the anther of *O. grandiflora*. He found that the somatic nucleus contained chromatic bodies which frequently approximated to the $2n$ number, i.e. 14. He suggested that they probably represented prochromosomes. In a later paper, Gates and Thomas (1914) found "light areas" in the chromosomes either at the middle or near the end of a chromosome. That most of these light areas correspond to the constriction region of the chromosome has been discovered quite recently. Hance (1918) made a detailed study of the somatic chromosomes of *O. scintillans*, another trisomic mutation of *O. Lamarckiana*, and found the longest chromosome to be nearly 1·8 times the length of the shortest. Levitsky (1931) concluded that such variation in the dimensions of chromosomes was due to the fact that Hance had made the measurements from drawings, i.e. the projection images of the chromosomes, at the same time disregarding the position of the chromosomes in relation to the optic plane. Van Overeem (1922) classified into four groups the chromosomes of *O. Lamarckiana*.

De Vries and Boedijn (1923, 1924), while trying to analyse the chromosomes of *O. Lamarckiana* and several of its mutations, drew conclusions indirectly as to their lengths. Levitsky (1931) measured carefully, according to his own devised method, the lengths of the chromosomes and concluded that *O. Lamarckiana* shows insufficient length differences. The dimensions vary gradually, the smallest differing from the largest by 1·35 times. He, however, found conspicuous differences in the shape of the chromosomes and classified them into four groups. The first consists of three pairs of more or less equal-armed chromosomes, the second of two sharply unequal-armed pairs and the third of one pair of unequal-armed chromosomes, but of smaller size. The fourth, a most conspicuous pair with a big appendage at one end of an arm separated by an achromatic zone. Darlington (1931) published drawings of the somatic chromosomes of a few species and hybrids of *Oenothera*. He found an unusual complexity of the visible structure of the chromosomes. They could not be paired. He suggested that the complicated behaviour of the chromosomes was due to the extreme heterozygosity of the species. Catcheside (1932), while comparing the somatic chromosomes of a haploid *O. blandina* and the normal diploid, found that there is a clear correspondence of chromosome types in the two. Marquardt (1938) has made some critical observations regarding the morphology of the somatic chromosomes of *O. Hookeri*. He, however, based his observations on nuclei undergoing mitotic divisions in anther tissue.

OBSERVATIONS

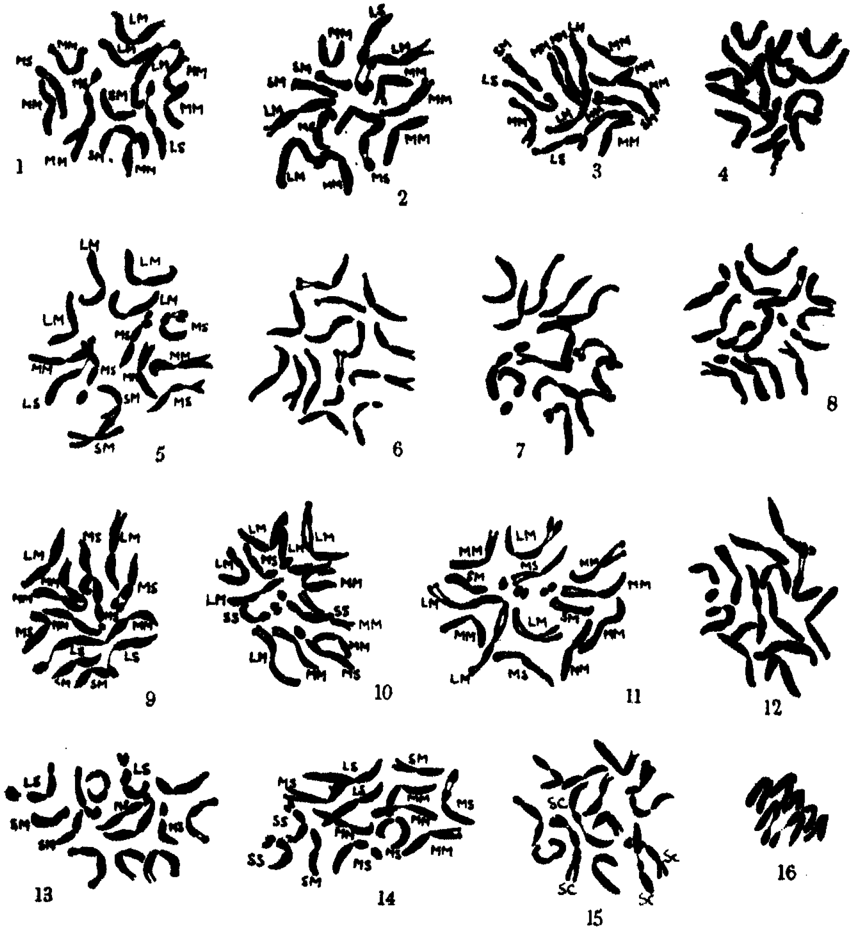
The diploid number in all the species and hybrids examined is fourteen, the stable number of the genus. Exceptionally, however, in some root-tip cells of *O. blandina*, fifteen chromosomes have been clearly counted (figure 4). The presence of this extra chromosome has been ascertained to be due to real fragmentation and is not an artefact. Though a large number of metaphase plates have been examined for each species, in no other case has fragmentation of chromosomes been observed. It appears further that this fragment belongs to one of the long chromosomes to which it is normally attached by a secondary constriction (figures 3 and 17).

Though the chromosomes of *Oenothera* species are very short (3·8–2·5μ), a marked difference in length between the longest and the shortest chromosomes, as will appear from table 1, has been observed for each species and hybrid.

TABLE 1

Name of the species and hybrids	Measurements of longest and shortest chromosomes in μ
O. Hazelae var. *parviflora*	3·5–2·5
O. angustissima var. *quebecensis*	3·6–2·6
Oenothera St Jerome	3·6–2·5
Oenothera sp. from Saskatchewan	3·8–2·5
O. Lamarckiana	3·8–2·5
O. blandina	3·8–2·5
O. biformiflora var. *cruciata* × *O. Lamarckiana*	3·8–2·6

De Vries and Boedijn (1923) found the range of chromosome length to be 15 to 6 units in *O. Lamarckiana*. Hance (1918) calculated it for *O. scintillans* as varying from 4·7 to 2·6 units. Darlington (1931) found the limits from 2·5 to 1·8μ to be general for *Oenothera* species. According to Levitsky (1931) they are between 2·7 and 2·0μ in *O. Lamarckiana*. Catcheside (1932), on the other hand, finds them to be between 3·6 and 2·8μ in *O. blandina*. It will be seen from table 1 that the measurements correspond fairly to those observed by Catcheside. Whatever be the cause of



FIGURES 1–16. Metaphase plates from different species and hybrids of *Oenothera* showing the number and morphology of the somatic chromosomes (see table 3). 1–2. *O. Lamarckiana*, note the heteromorphic pair of chromosomes *LM* and *LS*. In figure 2, only three chromosomes with secondary constrictions are distinguishable. 3–4. *O. blandina*, note 15 chromosomes including the fragment *f* in figure 4. The fragment probably belongs to the chromosome lying left of it. 5. *O. angustissima* var. *quebecensis*, note the *LS* chromosome with the big appendage. 6. *O. Hookeri*, two pairs of chromosomes with secondary constrictions. Note that some other chromosomes have terminal granules. 7–8. *O. ammophiloides* var. *laurensis*. 9. *O. Hazelae* var. *parviflora*. 10. *Oenothera* sp. from Saskatchewan. 11. *Oenothera* St Jerome. 12. *O. biformiflora* var. *cruciata*. 13. *O. Hazelae* (from Lockeport, N.S.). 14. *O. biformiflora* var. *cruciata* × *O. Lamarckiana*. 15. *O. missouriensis*. *Sc* = chromosome with secondary constriction. 16. Somatic anaphase in *O. blandina*; note the long pair of chromosomes with marked secondary constrictions. × 2800.

this anomaly in the measurements of different authors, it can be safely concluded from the present observations that the shortest chromosomes differ appreciably in length from the longest ones and this difference is fairly constant in all the species and hybrids examined.

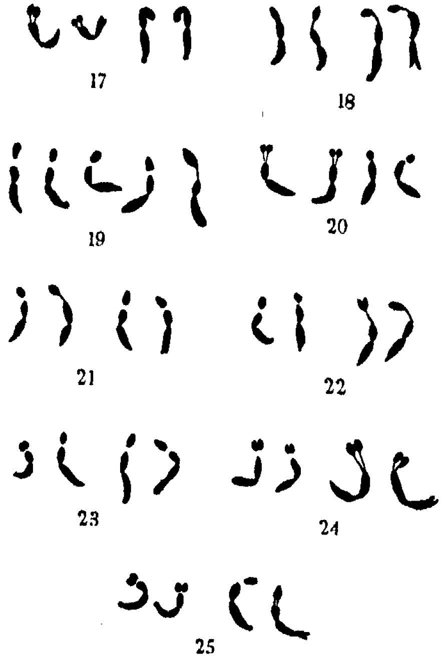
There is a distinct gradation in sizes of the chromosomes in *Oenothera*. The intermediate-sized chromosomes between the two extremes merge on either side, sometimes making their separation as a distinct group impracticable. This can be verified from table 2.

TABLE 2. SHOWING THE MEASUREMENTS OF ALL THE FOURTEEN CHROMOSOMES IN SOME SPECIES

	Measurements in mm. of individual chromosomes taken from camera lucida drawings													
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
O. Lamarckiana	18·5	18·5	17·5	17·5	15	15	14	13	13	13	12	12	10	10
O. blandina	18	18	17	16	15	15	13	13	13	13	12	11	12	10
O. Hazelae	18	18	16	16	15	15	14	14	13	13	12·5	12·5	11	11

The primary constriction in a chromosome of *Oenothera* is less conspicuous than the secondary constriction. The latter is remarkably big and the shorter arm of the chromosome (the appendage) separated by a secondary constriction sometimes gives the false appearance of a chromosome fragment. Unlike other species and hybrids, the secondary constrictions in one pair of chromosomes of *O. Hookeri* are very short (figures 6, 20). Besides the primary and secondary constrictions, some chromosomes often show other structural peculiarities. There are sometimes well differentiated globular bodies at either end of a chromosome (figures 6, 13, 15). This is especially clear in preparations stained with gentian violet. These bodies correspond with the terminal granules frequently observed at the ends of chromosomes in diakinesis (figures 64, 65, 74). The very early separation of the chromatids, especially at the ends of a chromosome, often gives the false appearance of a split trabant (figures 8, 10, 13). This peculiarity is commonly met with during the late prophase stages as well as in metaphase chromosomes. Besides the aforesaid peculiarities, one or more light areas in the body of the chromosome (especially with gentian violet staining) sometimes gives a misleading appearance of minute constrictions. The last two kinds of peculiarities are, however, not constant. Such complicated appearances of chromosomes have given rise to conflicting ideas regarding the interpretation of the morphology of the somatic chromosomes in *Oenothera*.

The marked secondary constrictions consist of two filaments representing the continuation of the chromatids (figures 30, 39). The appendage which is fixed to the arm of a chromosome by a long filament is, in most of the species, fairly big. The filament gives a positive Feulgen reaction in the same manner as the body of the chromosome. The general appearance of the secondary constriction varies considerably. Sometimes in the same section one finds, for instance, the length of the filament varying just as the length of the chromosome does. This is obviously due to shrinkage or swelling effect produced by the fixatives. After examining the chromosomes in different species of *Oenothera*, it becomes impossible to maintain a sharp distinction between a chromosome with secondary constriction and a satellited chromosome (figures 17–25).



Figures 17–25. Showing the comparative morphology of the chromosomes with secondary constrictions in different species and hybrids of *Oenothera*. 17. *O. blandina*, note the two homomorphic pairs. 18. *O. Lamarckiana*, note the heteromorphic pair of long chromosomes. 19. *O. angustissima* var. *quebecensis*; one of the five chromosomes has a very big appendage and a long secondary constriction. 20. *O. Hookeri*. 21. *O. ammophiloides* var. *laurensis*; first pair seems to be heteromorphic. 22. *O. Hazelae* var. *parviflora*. 23. *Oenothera* sp. from Saskatchewan. 24. *Oenothera* St Jerome. 25. *O. biformiflora* var. *cruciata* × *O. Lamarckiana*. × 2800.

After careful examination of a large number of plates and after using

Feulgen-light green staining it was found that the chromosomes in each species can be classified into groups according to their shapes and sizes and the position of the primary and secondary constrictions. The following groups of chromosomes have thus been identified; long, medium and short, with median or submedian primary constrictions and chromosomes possessing secondary constrictions. The general idiogram determined for each species and hybrid as well as the morphology of the chromosomes with secondary constrictions is shown in table 3.

It will be observed from table 3 as well as from table 4 that there are four chromosomes with secondary constrictions in all the species and hybrids examined, except in *O. angustissima* var. *quebecensis* where the number is five. These four chromosomes can be paired into two types in most of the species (figures 17–25). Not infrequently the two chromosomes appearing as a homologous pair differ appreciably in the respective lengths of their arms (cf. table 2). Such differences are in some species fairly constant, especially in chromosomes with secondary constrictions (figures 17, 22, 23) and lead to the view that corresponding chromosomes in the two complexes of a species may differ in length. In *O. Lamarckiana* the two long chromosomes forming a pair are distinctly heteromorphic (figures 2, 18). In *O. ammophiloides* the four chromosomes look alike at first but it can be seen that three have submedian primary constrictions and one a median. It appears that this species has also a heteromorphic pair of chromosomes (figures 7, 8 and 21). In *O. angustissima* var. *quebecensis* again, besides the two pairs of chromosomes, the fifth one, *LS*, has a big appendage separated by an unusually big secondary constriction (figures 5, 19). In *O. blandina* and *O. Hookeri* the four chromosomes form two distinct pairs (figures 17, 20) but in the former species one chromosome appears distinctly shorter than its partner. The appendages in this pair of chromosomes also look like trabants. These two pairs of chromosomes correspond to two pairs of nucleoli present in the somatic nuclei of *O. blandina* which remain attached to the fused nucleolus during somatic prophase (figure 28). It seems therefore probable that the supernumerary constrictions observed by Catcheside (1932) in this plant do not represent true secondary constrictions. In *O. Hookeri* one pair of chromosomes have remarkably big secondary constrictions whereas in the other pair they are hardly distinguishable (figures 6, 20). These four chromosomes can be identified precisely from the rest during prophase, where they only remain attached to the fused nucleolus by their secondary constrictions (figure 30).

It will be observed from table 4 that the maximum number of nucleoli in the root-tip nuclei corresponds to the number of chromosomes with

Table 3. SHOWING THE MORPHOLOGY OF THE CHROMOSOMES IN SOME OF THE DIFFERENT SPECIES AND HYBRIDS OF *OENOTHERA*

Species	General idiogram	Morphology of chromosomes with secondary constrictions	Figures
O. Hazelae var. *parviflora*	$2LM+2LS+4MM+4MS+2SM$	$2LS$ and $2MS$. One MS chromosome longer than its partner	9, 13, 22
O. angustissima var. *quebecensis*	$4LM+1LS+3MM+4MS+2SM$	$1LS$ and $4MS$. The appendage and constriction in the LS chromosome are very big	5, 19
Oenothera Moose Jaw, Sask.	$6LM+4MM+2MS+2SS$	$2MS$ and $2SS$. One SS chromosome longer than its partner	10, 23
Oenothera St Jerome	$4ML+6MM+2MS+2SM$	$2LM$ and $2SM$	11, 24
O. Lamarckiana	$3LM+1LS+6MM+2MS+2SM$	$1LM$, $1LS$ and $2MS$. The secondary constriction in one of the MS chromosomes more marked than in its partner	1, 2, 18
O. blandina	$2LS+2LM+8MM+2SM$	$2LS$ and $2SM$. The appendages of the SM chromosomes appear like satellites	3, 17
O. biformiflora var. *cruciata* × *O. Lamarckiana*	$2LS+4MM+4MS+2SM+2SS$	$2MS$ and $2SS$. The appendages of SS chromosomes look like satellites	14, 25

LM Long chromosome with median primary constriction.
MM Medium chromosome with median primary constriction.
SM Short chromosome with median primary constriction.
LS Long chromosome with submedian primary constriction.
MS Medium chromosome with submedian primary constriction.
SS Short chromosome with submedian primary constriction.

TABLE 4. SHOWING THE RELATIONSHIP OF THE CHROMOSOMES TO NUCLEOLI IN DIFFERENT SPECIES AND HYBRIDS OF *OENOTHERA*

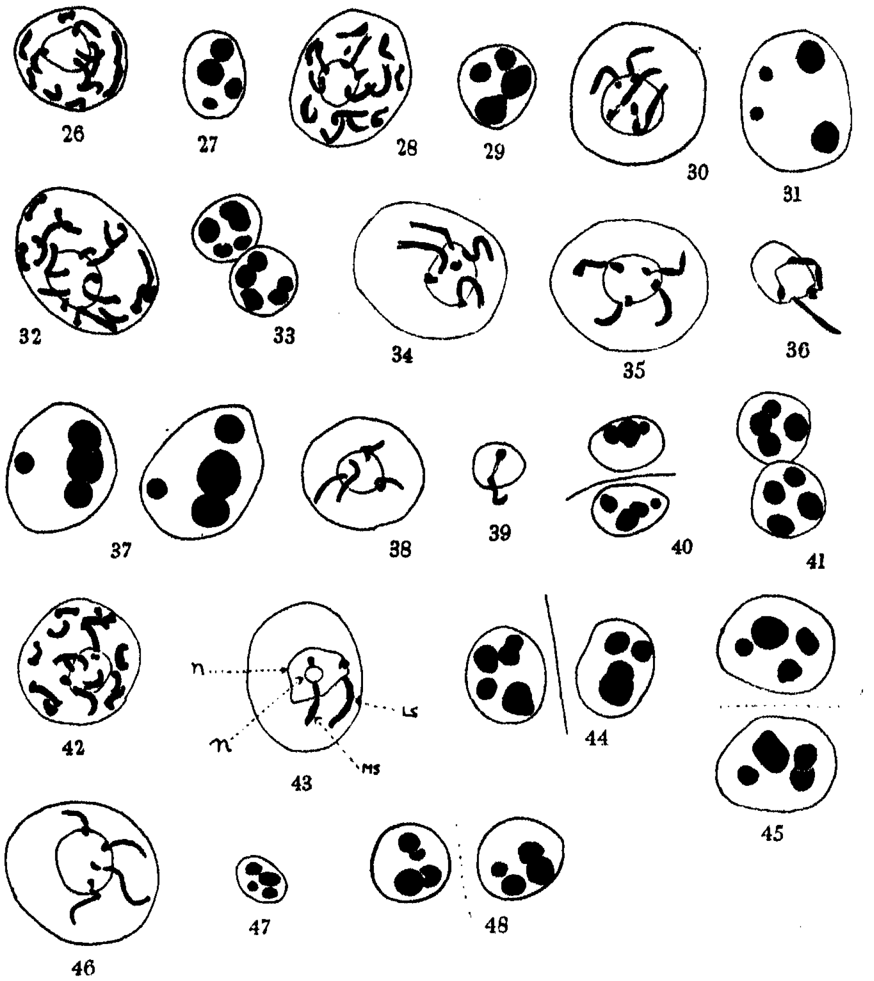
No.	Species	Culture no. (1938)	Maximum catenation	No. of chromosomes with secondary constrictions	Maximum no. of nucleoli in somatic nuclei	Size differences of nucleoli in somatic nuclei	No. of chromosomes attached to nucleolus during somatic prophase	No. of nucleoli and their sizes during diakinesis	Figures
1	*O. Hazelae* var. *parviflora*, from Port George, N.S.	101	(14)	4	4	1 big, 1 small, 2 intermediate	4	1 big, 1 small	13, 22, 42, 43, 44, 49
2	*O. Hazelae*, from Lockeport, N.S.	111*a*	(14)	4	4	1 big, 1 small, 2 intermediate	4	1 big, 1 small	9, 50
3	*Oenothera* St Jerome, Quebec	117	(14)	4	4	1 big, 1 small, 2 intermediate	4	1 big, 1 small	12, 24, 35, 37, 55*
4	*Oenothera*, sp., from Moose Jaw, Sask.	94	(14)	4	4	1 big, 1 small, 2 intermediate	4	2 big, 1 small	10, 23, 34, 53, 54*
5	*O. ammophiloides* var. *laurensis*	138	(14)	4	4	1 big, 1 small, 2 intermediate	4	1 big, 1 small	7, 8, 21, 38 to 41, 57, 58
6	*O. biformiflora* var. *cruciata*	141	(14)	4	4	1 big, 1 small, 2 intermediate	4	1 big, 1 small	12, 48*
7	*O. biformiflora* var. *cruciata* × *O. Lamarckiana*	204, Type I 204, Type II	(10), (4) (8), (4)	4	4	1 big, 1 small 2 intermediate	4	1 big, 1 small	14, 25, 46, 47, 59 to 63*
8	*O. Lamarckiana*	—	(12) 1_{II}	4	4	1 big, 1 small, 2 intermediate	4	1 big, 1 small	1, 2, 18, 26, 27
9	*O. blandina*	—	7_{II}	4	4	2 pairs, one pair smaller than the other	4	—	3, 17, 28, 29
10	*O. Hookeri*	—	7_{II}	4	4	2 pairs, one pair smaller than the other	4	—	6, 20, 30, 31
11	*O. missouriensis*	169/39	7_{II}	4	4	1 big, 1 small, 2 intermediate	4	—	15, 45
12	*O. angustissima* var. *quebecensis*	136	(14)	5	5	All different	5	—	5, 19, 32, 33

* Catenation determined for the first time.

secondary constrictions; this number being four in all the species and hybrids examined except in the case of *O. angustissima* var. *quebecensis* where the number is five. It is very difficult to determine this maximum number of nucleoli in small nuclei like those of *Oenothera*, because they generally fuse in early telophase and the number which is seen in later stages does not represent the maximum number for the species. Besides, in gentian violet preparations the small chromatic bodies representing the heterochromatic portions of chromosomes take up the stain similarly to nucleoli. During early stages, when the nucleoli are still very small, it becomes very difficult to distinguish between the two, thereby making the observation with this stain quite unreliable. With Feulgen-light green stain, however, the maximum number could be determined with fair accuracy during the early telophase when the young nucleoli lie separate from each other.

A distinct and constant size variation of the nucleoli is present in each species and hybrid. It will be noticed from table 4 and figures 27, 29, 31, 33, 37, 40, 41, 45, 47 and 48, that in all the species and hybrids examined, except *O. Hookeri* and *O. blandina*, there are one big, one small and two intermediate sized nucleoli. In *O. Hookeri*, there is one big pair and one very small pair of nucleoli, corresponding to the two different pairs of chromosomes with secondary constrictions (figures 6, 20, 31). In *O. blandina* also, there are two sizes of nucleoli corresponding to two pairs of chromosomes with secondary constrictions (figures 3, 17, 29).

Although it was not possible to trace the origin of the four nucleoli in reference to particular portions of chromosomes in every case, their origin at the secondary constrictions could easily be inferred from the study of the nature of attachment of particular chromosomes to the nucleoli during prophase. In figure 43 it will be seen clearly that one small nucleolus, lying in the region of secondary constriction of a chromosome, just fitted in as if indicating its point of origin. It will be observed further from table 4 that the number of chromosomes attached to the nucleoli corresponds to the maximum number of nucleoli and the number of chromosomes with secondary constrictions present in that particular species. As the chromosomes with secondary constrictions alone remain attached to the fused nucleolus during prophase they can be identified precisely from the rest in respect to the metaphase chromosomes (figures 28, 30, 32, 34, 35, 42 and 46). As we know that nucleoli are organized only at secondary constrictions, a count of the number of chromosomes attached to the nucleolus is always an important guide for establishing the maximum number of nucleoli in a species.

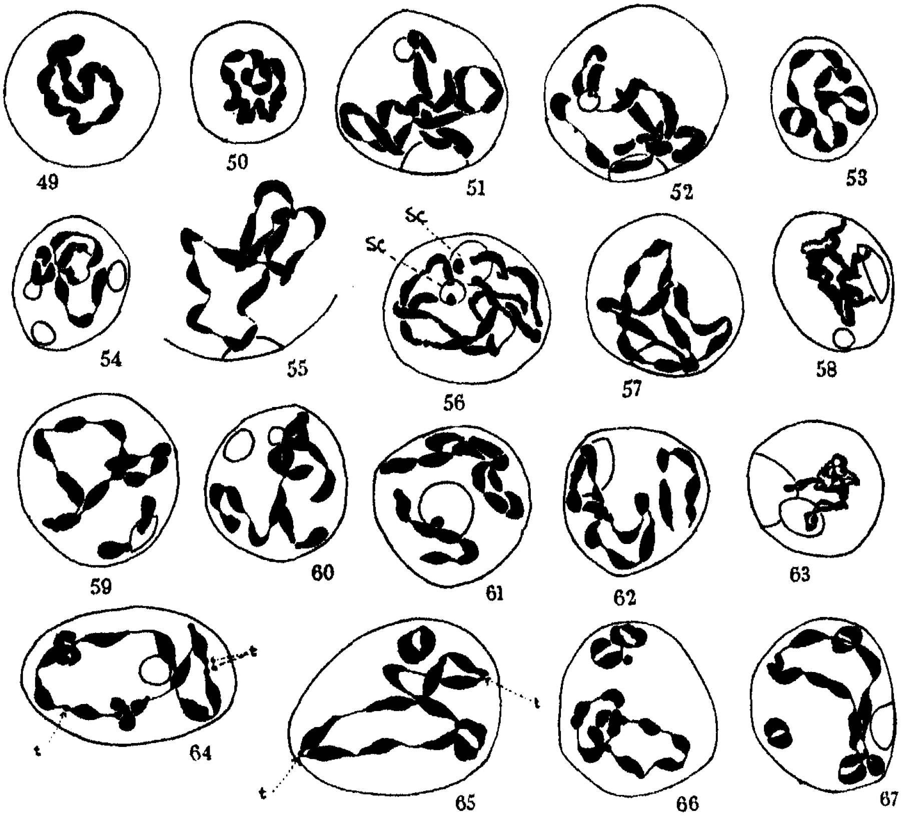


FIGURES 26–48. Showing attachment of chromosomes to the fused nucleolus during prophase and number and sizes of nucleoli during telophase in somatic nuclei of different species and hybrids of *Oenothera*. 26–27. *O. Lamarckiana*. 26. Four chromosomes attached to the nucleolus and the remaining ten lying free. 27. One big, one small and two intermediate nucleoli. 28–29. *O. blandina*. 28. Four chromosomes attached and the remaining ten lying free. 29. Two pairs of nucleoli. 30–31. *O. Hookeri*. 30. The two pairs of chromosomes attached. 31. Two pairs of nucleoli. 32–33. *O. angustissima* var. *quebecensis*. 32. The five chromosomes attached and the remaining nine lying free. 33. Five nucleoli of different sizes. 34. *Oenothera* sp. from Saskatchewan. 35–37. *Oenothera* St Jerome. Note in figure 36, one chromosome showing the long filament on the surface of the nucleolus. 38–41. *O. ammophiloides* var. *laurensis*. In figure 39, the secondary constriction of the chromosome attached to the nucleolus appears to consist of two filaments. 42–44. *O. Hazelae*. In figure 43, the small nucleolus at the secondary constriction can still be distinguished from the other fused nucleolus. *n* = nucleolus; *MS* = medium chromosome with submedian primary constriction. *LS* = long chromosome with submedian primary constriction. 45. *O. missouriensis*. 46–47. *O. biformiflora* var. *cruciata* × *O. Lamarckiana*. 48. *O. biformiflora* var. *cruciata*. × 2000.

A definite relationship between the sizes of the nucleoli on the one hand and the dimensions of the appendages or the lengths of filament on the other, could not be established during the present study, although variation in all these characters is very conspicuous in most of the species. Lesley (1938), in tomato, has found that an increase in the dimensions of the satellite is accompanied by an increase in the dimensions of the nucleolus. It seems very likely that the size of the nucleolus is dependent upon the specific capacity of the chromosomes to produce a definite amount of nucleolar substance and not merely on the length of the filament or dimensions of the satellite.

Due to the marked condensation of the chromosomes during diakinesis and the general swelling caused by the fixatives, the secondary constrictions of chromosomes become obscure at this stage. The tiny globular bodies at the ends of the chromosomes, the terminal granules, often give the misleading appearance of trabants but are much smaller than the big appendages and are not separated by the marked secondary constrictions so characteristic of the somatic chromosomes of *Oenothera*. Clear cases showing secondary constrictions as well as the attachment of the chromosomes to the nucleoli have been observed in a few cases (figures 52, 56, 68, 70, 74). Although four chromosomes with secondary constrictions cannot be identified during diakinesis, indirect evidence shows that the conditions are similar in the pollen mother cells to those in the somatic nuclei. From table 4 it will be seen that the number of nucleoli during diakinesis does not correspond to the maximum number four. This is due to fusion of nucleoli prior to diakinesis. In most of the cases two nucleoli, one big and one small were detectable (figures 52, 56, 58, 60, 63, 71, 72), although in some, three nucleoli of different sizes were observed (figures 54, 78). The presence of two nucleolar bodies in one nucleolus (figures 79) also confirms the view that the particular nucleolus is a fusion product of at least two separate nucleoli. It appears therefore that the two nucleoli commonly seen during diakinesis really represent the fusion of four nucleoli corresponding to the four chromosomes with secondary constrictions.

A nucleolus takes up a particular position in reference to the ring of chromosomes. In *O. Hazelae* for instance one small chromosome with a split satellite always remains attached to a small nucleolus (figure 52). A systematic study of the position of nucleoli with reference to the catenations could not be made during the course of this investigation, as the catenation study was chiefly made from smear preparations, stained with gentian violet, in which the nucleoli are not properly stained. Another difficulty encountered was the frequent appearance of the nucleoli lying



FIGURES 49–67. 49–52. *O. Hazelae.* 49 and 50. Showing (14) in two different strains. 51. Two nucleoli, one big and one small at different positions in the broken ring. 52. One small chromosome attached to the small nucleolus by a split satellite. 53–54. *Oenothera* sp. from Saskatchewan. 53. (14). 54. Three nucleoli of different sizes, two attached and one free. 55–56. *Oenothera* St Jerome. 55. (14). 56. One big and one small nucleolus: chromosomes with secondary constrictions (*Sc*) attached to two nucleoli. 57–58. *O. ammophiloides* var. *laurensis.* 57. (14). 58. One big and one small nucleolus; note the change in the shape of the big nucleolus. 59–63. *O. biformiflora* var. *cruciata* × *O. Lamarckiana.* 59. Type II, showing (8), (4), 1_{II}. The free pair is attached to the nucleolus. 60. Type II; the small nucleolus appears to be attached to the chain of four, the other nucleolus lying free. 61. Type I; nucleolus attached to chain of four, the dark body on the nucleolus is the nucleolar body. 62. Type I, (10), (4); the fused nucleolus attached to (10). 63. Two pairs of chromosomes attached to nucleoli, one big and one small. 64. *O. biformiflora* × *O. Lamarckiana,* showing (10), 2_{II}; t = terminal granule. Nucleolus attached to (10). 65. *Oenothera* St Jerome × *O. Lamarckiana,* showing (10), 2_{II}. 66. *O. deflexa* var. *bracteata* × *O. Lamarckiana* showing (6), (4), 2_{II}. 67. *O. deflexa* × *O. Lamarckiana* showing (10), 2_{II}. × 2000.

appressed to the nuclear membrane and quite apart from the chromosomes (figures 60, 67, 78). This appearance, however, is due to fixation effects, which can be verified by examination of a large number of nuclei.

The catenation was determined in a number of species and hybrids. The results are presented in table 5.

A single trisomic mutation of *O. Hazelae* var. *parviflora*, arose in one culture in 1938. The plant was dwarf with markedly narrower leaves and flowers smaller than the parent type. Several flowers were selfed but none set seeds. The chromosome number was found to be fifteen (figures 75, 77, 80). During meiotic prophase in the pollen mother cells of this mutant two nucleoli, one big and one small, have been found to be a general condition, as in the parent. In exceptional cases, however, three nucleoli were observed (figure 78). There is generally a small spherical, deeply stained body on the surface of the nucleolus, representing the nucleolar body or endonucleolus. Sometimes two such bodies are present in one big nucleolus (figure 79). Irregular distribution of the univalents during first meiotic anaphase has been observed in a few cases, but the general condition is an eight and seven distribution to the two poles (figure 80). The catenation of chromosomes during diakinesis is very irregular. The maximum is a chain of fifteen, though catenations showing $14+1$, $11+2_{II}$; $11+4$; etc., have been observed (figures 75, 76, 77). Non-disjunction in the pollen mother cells leading to eight-and-six distributions has been observed quite frequently in the parent *O. Hazelae* (figure 81).

In one batch of root-tips of *O. Hazelae* the chromosomes at metaphase appeared to show somatic pairing. Sometimes each pair was found to remain attached at one end (figure 82). It was found subsequently from well fixed material that this pairing was only chance association brought about by bad fixation. Marquardt (1938) has observed similarly in the anther archesporium of *O. Hookeri*, cases of apparent pairing of somatic chromosomes. He concluded that the mutual co-ordination between chromosomes was not so marked as to be described as regular somatic pairing. His suggestion that this association of the chromosomes may represent a step towards conjugation, which will take place in the following nuclear division, does not appear to be sound from the present observation.

Distinct chromatic bodies (prochromosomes) scattered about in the resting nuclei of root-tip cells have been observed in all species of *Oenothera*. The number of these bodies corresponds to the number of chromosomes (figure 83) and they are Feulgen positive. Some of them, generally four, remain in close association with the nucleolus. Doutreligne (1933) showed that these bodies are particular portions of chromosomes representing the

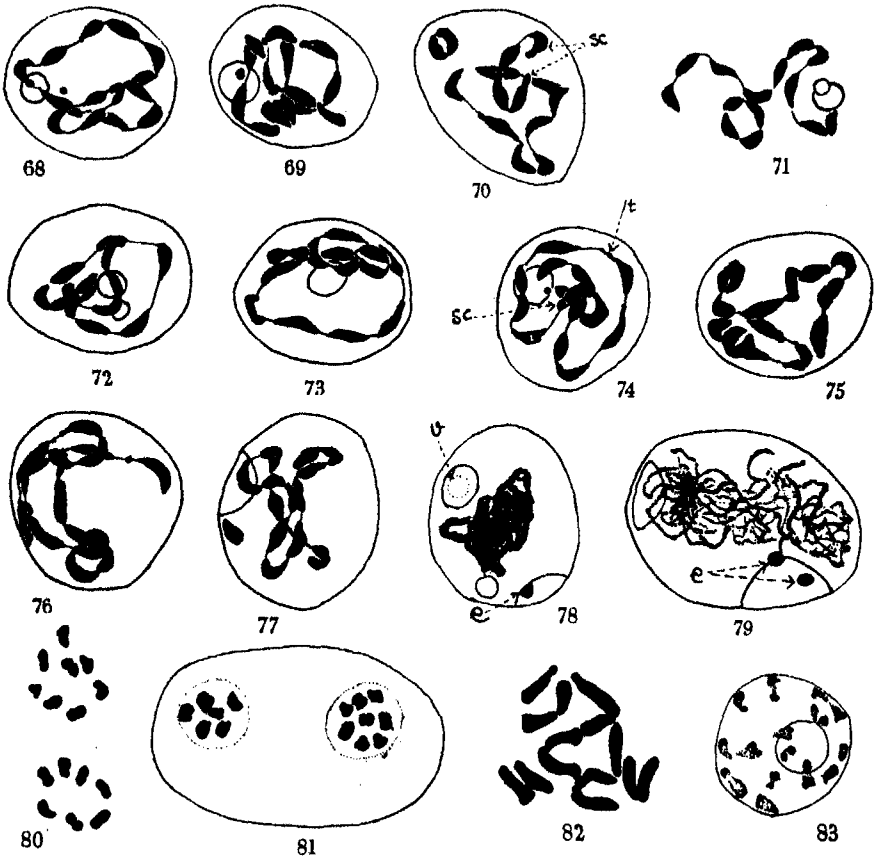
TABLE 5. CATENATIONS OF SOME INTERSPECIFIC HYBRIDS AND ONE TRISOMIC MUTATION OF *OENOTHERA*

No.	Name of the parent species	Culture no. (1938)	Principal distinguishing characters of the segregate types in F_1	Maximum catenation	Figure no.
1	*O. biformiflora* var. *cruciata* × *O. Lamarckiana*	204*	Type I†	$(10)+(4)$	61, 62
2	*O. biformiflora* var. *cruciata* × *O. Lamarckiana*	204.I.9*	Type I†	$(10)+(4)$	
3	*O. biformiflora* var. *cruciata* × *O. Lamarckiana*	204	Type II‡	$(8)+(4)+1_{II}$	59
4	*O. Lamarckiana* × *O. biformiflora* var. *cruciata*	205*	Type II‡	$(8)+(4)+1_{II}$	
5	*O. biformiflora* × *O. Lamarckiana*	206	Type I†	$(10)+2_{II}$	64
6	*O. biformiflora* × *O. Lamarckiana*	206	Type II‡	$(10)+2_{II}$	
7	*O. Lamarckiana* × *O. biformiflora*	207	Type II‡	$(8)+(4)+1_{II}$	
8	*O. deflexa* × *O. Lamarckiana*	213	Type II‡	$(6)+(4)+2_{II}$	66
9	*O. deflexa* var. *bracteata* × *O. Lamarckiana*	214	Type II‡	$(10)+2_{II}$	67
10	*O. pycnocarpa* var. *parviflora* × *O. Lamarckiana*	222	Type II‡	$(10)+2_{II}$	73
11	*O. pycnocarpa* var. *cleistogama* × *O. Lamarckiana*	224	Type I†	$(10)+(4)$	68
12	*O. pycnocarpa* var. *cleistogama* × *O. Lamarckiana*	224	Type II‡	$(8)+(4)+1_{II}$	
13	*Oenothera* St Jerome × *O. Lamarckiana*	209	Type II‡	$(10)+2_{II}$	65
14	*O. Hazelae* (trisomic mutation)	102.I.1		15; $14+1$; $13+1_{II}$	75 to 77

* All the plants belonging to cultures 204 and 205 had cruciate flowers except one plant (204.I.9) which produced flowers with three types of petals, broad, cruciate and intermediate.

† Type I = leaves with white midrib.

‡ Type II = leaves with pink midrib.



FIGURES 68–83. 68–72. *O. pycnocarpa* var. *cleistogama* × *O. Lamarckiana*. 68. Type I, showing (10), (4), one chromosome attached to the nucleolus by a secondary constriction. 70. Type II; (8), (4), 1_{II}. Two chromosomes with secondary constrictions placed end to end in the chain of four. 71. Type I, one big and one small nucleolus fusing. 72. Type I; the small nucleolus attached to chain of four and the big one to (10). 73. *O. pycnocarpa* var. *parviflora* × *O. Lamarckiana*, showing (10), 2_{II}; the nucleolus attached to (10). 74. (10), (4), in *O. pycnocarpa* var. *cleistogama* × *O. Lamarckiana*, Type I; the (4) is broken. 75–80. Trisomic mutation of *O. Hazelae*. 75. $11+2_{II}$. 76. Chain of 15. 77. 14+1. 78. Three nucleoli; $e=$ nucleolar body, $v=$ vacuole. 79. Zygotene; one nucleolus with two nucleolar bodies. 80. 7 and 8 distribution during anaphase I. 81. Metaphase II in pollen mother cells of *O. Hazelae* showing 8 and 6 grouping due to non-disjunction. 82. False somatic pairing. 83. Prochromosomes, stained with Feulgen; four of them in close association with the nucleolus. Root-tip cell of *Oenothera* sp. from Saskatchewan. Figures 68–81, × 2000; figures 82–83, × 2800.

regions containing the spindle attachment constrictions. The presence of distinct chromatic bodies in the resting nuclei of somatic cells was observed long ago by Davis (1909) in *O. grandiflora* and by Gates (1912) in *O. lata*. Marquardt (1938) has shown in *O. Hookeri* that these bodies are the heterochromatic middle portions of chromosomes, as compared to the euchromatic end segments, which following condensation give the typical appearance of chromocentres during resting stage.

DISCUSSION AND GENERAL CONCLUSIONS

Fragmentation of somatic chromosomes in *Oenothera* has until now been reported only in some of the mutations of *O. Lamarckiana* (Lutz, 1916; Hance 1918; Van Overeem 1922). It is now realized that part of the observation regarding fragmentation by previous authors was due to misinterpretation of the remarkably big secondary constrictions so characteristic of *Oenothera*. It is quite certain, however, that there must be real fragmentation as well to account, for instance, for the observations of Hance (1918) who counted 21 chromosomes in some of the somatic cells of *O. scintillans*. Taking into consideration that fragmentation has not been observed in any other species or hybrids but in *O. blandina* and the previous observations of fragmentation in some mutations of *O. Lamarckiana*, it appears very likely that this phenomenon expresses a peculiar condition of certain chromosomes in mutations of *O. Lamarckiana*, probably indicating weak points in those chromosomes where they can break.

It has been shown already that in most species and hybrids of *Oenothera*, there are four chromosomes with secondary constrictions corresponding to four nucleoli in the root-tip cells. The extraordinary situation in *O. angustissima* var. *quebecensis*, where there are five secondary constrictions and five nucleoli, can be explained by the fact that this species shows certain morphological characters quite distinct from other Onagrads. In fact, as suggested by Gates and Catcheside (1932) and Gates (1933), this species shows characters suggesting an intermediate position between the two subgenera, *Onagra* and *Raimannia*. Very little information is available regarding the somatic chromosomes of any species belonging to the subgenus *Raimannia*. One can only make out from the figures given by Darlington (1931) for *O. Berteriana* that there are more than two, maybe four to seven, chromosomes with secondary constrictions. In the absence of cytological information on species of *Raimannia*, nothing can be said as to the origin of *O. angustissima* var. *quebecensis*. It will be very interesting to know in this connexion the conditions in such species as *O. campylocalyx* Koch and Bouché, which show distinct *Raimannia* characters.

It has been shown definitely that in *O. Hookeri*, like other species, there are two pairs of chromosomes with secondary constrictions corresponding to two pairs of nucleoli. Marquardt (1938), on the other hand, claims that there is only one pair of Sat-chromosomes in *O. Hookeri*. He has criticized the observations of Wisniewska (1935), who found three more pairs of chromosomes with a round appendage at the end of one arm, and he explains that such appendages are not a constant natural feature of a particular chromosome. He further claims that his conclusion is correct on the ground that he failed to observe such appendages in preparations stained with aceto-carmine, the latter being the classical method for distinguishing heterochromatin from euchromatin. It seems that Marquardt has failed to see the other pair of chromosomes with secondary constriction due to the fact that he based his observations on anther tissue, where it is well known that the fixation of the chromosomes is definitely poorer. He missed the pair of chromosomes with very short secondary constriction. In support of this conclusion, the observations of Catcheside (1932) can be cited. He found that the chromosomes in the anther tissue are much shorter and thicker than those in the root-tips. The problem of fixation in *Oenothera* has already been discussed.

It is interesting to learn that while a pair of chromosomes with secondary constrictions are heteromorphic in *O. Lamarckiana*, in *O. blandina*, a homozygous mutation of *O. Lamarckiana* without catenation, these chromosomes are homomorphic. One concludes that structural changes of chromosomes in *O. blandina* (probably due to segmental interchange) have not only brought back homozygosity in the species but also the morphological homology of a certain pair of chromosomes. The view that the heteromorphic nature of the Sat-chromosomes indicates heterozygosity of the individual seems to have obtained a direct proof from the evidence just put forward.

It is interesting further to note that in *O. Lamarckiana* the nucleoli are of different sizes and cannot be paired, while in *O. blandina* the nucleoli can be paired into two sizes corresponding to two pairs of chromosomes with secondary constrictions. Presence of unpaired nucleoli therefore indicates heterozygosity of the species. This is further substantiated by the fact that in *O. Hookeri*, a homozygous species without catenation, there are also two distinct pairs of nucleoli corresponding to two pairs of chromosomes with secondary constrictions. The rather extraordinary situation in *O. missouriensis* (without catenation) where the nucleoli could not be paired, cannot be explained at present till further cytological information is available. It must be pointed out here, however, that the roots from which the present observations are made, were collected from seeds produced by crossing two strains of *O. missouriensis*. The female parent shows no catenation (Hedayetullah 1933) and is completely self-sterile (Gates 1939).

The genetic significance of the number of nucleoli in plant tissue was first realized by De Mol (1926) who found that the number of nucleoli in *Hyacinthus* may be used as a guide to the number of chromosome sets. Kuhn (1928), after examining thirty-seven species of *Thalictrum*, found that the species have trabants. He concluded that univalent idiograms apparently have never more than one pair of trabants. These expectations took a positive aspect and harmonized into a general hypothesis since the discovery by Heitz (1931*a*) of the relation of Sat-chromosomes to the nucleolus. Heitz concluded that the nucleoli as a rule arise in telophase from the satellites in a particular chromosome pair. In a later paper (1931*b*), after examining thirty-three species of *Vicia*, he concluded that all plants probably have satellited chromosomes which give rise to nucleoli in telophase. The functional correspondence of the secondary constrictions to the satellites, in regard to the organization of the nucleoli, has been realized quite recently. In fact, a distinction between the two seems to be rather arbitrary than real. However, the presence of secondary constrictions which do not produce nucleoli has been recorded by a number of workers. Sato (1936, 1938) has found such a condition in *Scilla*, *Zephyranthes*, *Cyrtanthus*, *Hebranthus* and *Narcissus*. Sato (1937) and Resende (1937) in Aloinae, and Fernandes (1936) in *Narcissus*. Until more direct and exact information is forthcoming regarding the nature of the constrictions and their evolution in chromosome-segments, it seems useful to call all constrictions which are related to nucleolar organization "nucleolar constrictions".

McClintock (1931) independently came to the same conclusion as Heitz regarding the relation of Sat-chromosomes and the organization of the nucleoli in the case of maize. She concluded in a later paper (1934) that the number of nucleoli in somatic telophase is correlated with the number of Sat-chromosomes, haploid cells having one, diploids two and triploids three. She has made a very significant observation in maize that certain genomic deficiencies may lead to the formation of many small scattered nucleoli instead of a fixed number.

Quite an amount of literature has been accumulated on the subject since the discovery of the relation of Sat-chromosomes to nucleoli. The problem has been discussed by Gates (1937, 1938) quite recently from several aspects and need not be repeated here. Many observations, contradictory to the general principle, however, have been recorded quite recently, making the relation of chromosomes to nucleoli more complicated than was originally supposed. According to Bushnell (1936) in Labiatae and Okuno (1937) in *Lobelia*, there is sometimes no relation between the satellited chromosomes and the nucleoli. It appears that some such conflicting observations may be explained as partly due to inadequate technique and to the presence of extremely small trabants, as discovered in some *Trillium* species (Mensinkai 1939), *Haworthia* species and others. A judicious and wider application of the phenomena of amphiplasty as described by Navashin (1927, 1934) may also account for the apparent failure of the general principle of correspondence of the number of nucleoli to Sat-chromosomes in certain cases.

Though conflicting data are being put forward from time to time, the principle that primary diploids have a single pair of nucleoli seems to be a useful guide in the study of the evolution of the nucleus. In fact, many of these negative observations may ultimately help in establishing the hypothesis as a biological law, rather than to disprove it. Gates (1938) accordingly has pointed out that "Three complementary lines of observation can now be brought to bear on the phylogeny of the nucleus in cases of primary or secondary polyploidy. When these confirm each other, the evidence of former changes, even in the basic number of chromosomes, is on a firm basis."

The application of the principle in the case of *Oenothera* has met with considerable difficulty. Parthasarathy (1938) already doubted, without making any direct observation, that the rule of the correspondence of satellites and nucleoli might not be applicable to definitely known segmental interchanged heterozygotes, such as *Oenothera, Aucuba, Anthoxanthum*, etc. Contrary to such expectations, however, a close correspondence between the number of nucleoli and the number of secondary constrictions in *Oenothera* has been established for each species and hybrid of *Oenothera* studied. The problem still remains, to explain the presence of four nucleoli in both homozygous and heterozygous species of *Oenothera* which are regarded as true diploids.

McClintock (1934), in *Zea Mays*, has shown that a change in the number of nucleoli can be brought about in some of the progenies by X-raying the parent diploid plants. This she explained as due to breakage at the region of the nucleolar body of chromosome 6 followed by an interchange with chromosome 9, the two resulting chromosomes both having a bit of the nucleolar body. Both the chromosomes are therefore able to organize a nucleolus. Microspores of this plant, instead of possessing one chromosome with nucleolar body, possess two such chromosomes. Explanation of the four nucleoli in *Oenothera* on this basis seems to be unlikely.

It is now known that the linked condition of the chromosomes has arisen within the genus *Oenothera*. The subgenus *Onagra* began with large-flowered, homozygous species, with free chromosome pairs from which were developed species with progressively increasing chromosome catenation. The reverse process, by which more homozygous forms with no chromosome linkage arise as mutations from heterozygous ancestors, can also take place.

From the observations recorded in this paper it can be safely concluded that the constant presence of four nucleoli corresponding to four secondary constrictions in both large-flowered homozygous species with no chromosome linkage, such as *O. Hookeri*, *O. missouriensis* and *O. blandina*, as well as in the heterozygous species showing high catenation, such as *O. Lamarckiana*, *O. Hazelae*, *O. biformiflora cruciata* and *O. ammophiloides*, is a more primitive character than the linkage of chromosomes. It further shows, from the behaviour of *O. Lamarckiana* and *O. blandina*, that these two conditions are independent of each other in these species and most likely in others. As already pointed out, there is also reason to believe that *O. Berteriana*, the only representative of the subgenus *Raimannia* whose somatic chromosomes have been examined, also possesses not less than four secondary constrictions and probably the same number of nucleoli in its somatic nuclei, the maximum catenation for the species being a ring of fourteen. It can be reasonably concluded that four nucleolar chromosomes is the minimum number in the genus *Oenothera*.

Applying the principle of correspondence of the number of Sat-chromosomes to the number of nucleoli in relation to polyploidy, the problem turns to whether *Oenothera* should be looked upon as essentially a diploid or represents an exception to the principle already mentioned. Certain previous observations, mentioned below, along with the present observations give strong evidence in support of the view that seven may be a derived chromosome number. A very significant observation in this connexion was made by Emerson (1929), who found the almost constant presence of a bivalent and in one case a chain of four univalents in pollen mother cells of a haploid *O. franciscana*. Similarly, Catcheside (1932) has shown in haploid *O. blandina* that out of 1186 pollen mother cells 218 showed the presence of one bivalent and five univalents, five cells showing trivalent chromosomes and one a quadrivalent. He also recorded multivalent formation, such as trivalents and quadrivalents during diakinesis in diploid *O. blandina*. He has stressed that the trivalents formed from a bivalent and an univalent represent a true multivalent formation and cannot be interpreted as due to interlocking, etc. He holds that the presence of multivalents in haploid *Oenothera* proves the presence of reduplicated segments in non-homologous chromosomes. The evidence put forward in this paper indicates that the haploid number in *Oenothera* is a secondary number. Further cytological observations of this kind in homozygous species of *Oenothera* and in related genera, will show the exact basic number for the genus.

In conclusion I desire to express my profound gratitude to Professor R. R. Gates, F.R.S., under whose supervision the present work was carried out.

SUMMARY

1. A comparative study of the relation of chromosomes to nucleoli has been made in nine species and one hybrid of *Oenothera*. The distinction between a satellited chromosome and one with secondary constriction breaks down in *Oenothera*. Both the constricted region and the appendage are well marked in most of the species. The filament, like the appendage, is Feulgen-positive.

2. The chromosomes have been classified in some of the species according to their sizes, the situation of the primary constriction and the presence of secondary constrictions (table 3).

3. An exact correspondence between the number of secondary constrictions and the number of nucleoli has been established in each case. This number is four in all the species except *O. angustissima* var. *quebecensis* where it is five. Determination of the position of nucleolar chromosomes in the ring is important as a further means of identifying chromosomes in the two complexes of a species.

4. The presence of four nucleoli corresponding to four secondary constrictions in (*a*) heterozygous species with high chromosome catenation, such as *O. Lamarckiana*, *O. Hazelae* and *O. biformiflora* and (*b*) homozygous species with seven free pairs such as *O. Hookeri* and *O. missouriensis*, proves that the presence of four nucleolar chromosomes is an older character than chromosome linkage. This supports the view that ring formation in *Oenothera* has evolved in the genus.

5. That morphological changes of chromosomes can be brought about by segmental interchange is evidenced by the fact that at least one hetero-

morphic pair of Sat-chromosomes in *O. Lamarckiana* has become homomorphic in its mutant *O. blandina*.

6. There is variation in the sizes of the nucleoli in species and hybrids of *Oenothera*. *O. Lamarckiana* has one very small, one quite big and two intermediate nucleoli, whereas in the homozygous *O. blandina* and *O. Hookeri* there are two distinct pairs of nucleoli. An unpaired condition of nucleoli in *Oenothera* therefore indicates heterozygosity of the species.

7. Prochromosomes, which are Feulgen-positive, have been observed in somatic cells of some species.

8. The catenation of three species and nine interspecific hybrids has been determined (tables 4, 5). In a narrow-leaved trisomic mutation of *O. Hazelae* the 15 chromosomes showed irregularities in catenation.

9. The presence of four nucleoli corresponding to four secondary constrictions, the frequent presence of multivalent chromosomes in the pollen mother cells of haploid and diploid plants, as well as the fact that no common basic number is found in the family Onagraceae, indicates that the haploid number 7 in *Oenothera* is a derived number. On this assumption *Oenothera* species cannot, therefore, be looked upon as true diploids.

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On the hardening of the ootheca of *Blatta orientalis*

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The ootheca of *Blatta orientalis* was chosen as the object of an investigation into the hardening mechanism of the insect cuticle because the material of which it is composed looks like the hard brown exocuticle of a typical insect. It is, however, secreted by glands, from which comparatively large amounts of the substances concerned can be obtained.

The ootheca is about 8 mm. long, and has the shape of a carpet bag, opening by two flat adpressed lips, which have a regular pattern of ridges and hollows on them. Inside there are about a dozen elongated eggs, arranged in two rows. In the wall of the ootheca are embedded numerous crystals of calcium oxalate (Hallez 1909). The function, if any, of these crystals is obscure; they may help to harden the wall, or they may simply be excretory products, since Sinéty (1901) has recorded crystals of an oxalate from the Malpighian tubules of a phasmid.

The production of the ootheca occupies about 24 hr. at 27° C. Until it is complete it is carried by the female in the genital pouch: the end projects farther and farther as more is secreted, until finally it is dropped or buried by the insect. In the early stages of secretion the small portion of the ootheca which projects from the pouch is white and opaque, but in the course of 3 or 4 hr. it becomes transparent, and the colour changes, first to pink, and then to a reddish chestnut. After it is laid the ootheca continues to darken, and after about 3 weeks it becomes almost black. When white it is still soft, but it becomes progressively harder and more brittle. If the ootheca is removed from the pouch before it is completed it can be observed that there is an area at the end that is in process of secretion which is white and still very soft.

COMPOSITION OF THE OOTHECA

Campbell (1929) was the first author who remarked that the ootheca did not contain any chitin, since he found that it dissolved in hot caustic alkalies, leaving only an extremely thin membrane of unknown composition. The substance of the ootheca shows all the negative properties which, according to Wigglesworth (1933), are characteristic of the "cuticulin" of the exocuticle of *Rhodnius*. It is not digested by pepsin or trypsin, does not dissolve in dilute acids or alkalies, and is only very slowly attacked by concentrated hydrochloric acid in the cold. The reaction which it gives with Millon's reagent is typical; on warming there is first a strong red colour developed, but on further heating the red colour fades, and the whole structure melts to a viscous yellow fluid, which sets hard on cooling and forms a brittle yellow mass. This material is not soluble in alcohol, ether, carbon disulphide, or dilute acids, but it will dissolve in dilute alkalies, in which it gives a deep orange solution. Neither this material nor the original oothecae will stain with fat stains. The behaviour of the ootheca with dilute nitric acid, or with Schulze's mixture (potassium chlorate in nitric acid) is the same, except that there is no red colour developed.

Freshly darkened oothecae give a positive reaction with ninhydrin, and a positive biuret reaction if they are dissolved in strong caustic soda solution. Both these reactions are given very strongly by the newly secreted ootheca before it has darkened, but fall off with increasing age of the material. The argentaffin test (performed on sections) is positive. This test, elaborated by Lison (1936), is carried out by immersing sections in a dilute solution of ammoniacal silver oxide for 24 hr., when any sufficiently strong reducing agent present in the sections gives rise to a black precipitate of silver. The number of compounds which will yield a positive result is very limited; the only ones yet found in biological material are polyphenols and polyamines. The results of the test are not very strongly positive, but since the ootheca has to be treated with dilute hydrochloric acid to remove the crystals of oxalate before it can be sectioned at all, this is not necessarily very significant. If aqueous extracts of the oothecae are tested with dilute ferric chloride solution they give a brilliant green colour, which changes to red on the addition of sodium carbonate, thus showing the presence of ortho-dihydroxyphenols. From these results it appears that the ootheca is mainly composed of a protein, and that the darkening is probably due to the oxidation of an ortho-dihydroxyphenol.

The type of natural protein which most closely approaches the protein of the ootheca in the degree to which it resists chemical action is keratin. Since more is known about the structure of the molecule of keratin than is known about most scleroproteins, it is worth while enquiring into the factors to which keratin owes its stability, with the object of seeing whether the same conditions apply to the protein of the ootheca.

The work of Astbury and his collaborators (for summary see Astbury 1936) has made clear the main points in the structure of the keratin molecule. The resistant properties of keratin are largely due to the presence of primary valence cross-linkages between the protein chains, formed by the sulphur atoms in cystine side chains. The presence of this sulphur cross-linkage system is very characteristic of keratin, but it is not the only factor responsible for its stability. If keratin is treated with an alkaline solution of sodium sulphide, or other strong reducing agent, the sulphydryl groups are reduced, so that the cross-linkages are broken down, and the protein becomes soluble. Keratin dissolved in this way can be re-oxidized, so that the cystine bridges are reformed, but it never regains its former stability, and remains soluble in dilute alkalies and liable to hydrolysis by enzymes. This has been explained by Goddard and Michaelis (1934) as being due to the fact that the original parallel orientation of the chains is lost, so that close packing becomes impossible, and the effect of secondary valence bonds and of van der Waals's forces in keeping the chains together is very much reduced.

The oothecae of *Blatta* are isotropic when examined in polarized light, both in section and in surface view. This shows that there is no parallel orientation of the protein chains; a conclusion which is confirmed by an examination of the X-ray diffraction pattern. This shows a typical powder diagram, with a complete ring, whose distance from the centre corresponds to a spacing of 4·5 A. Since the oothecae are composed of a protein with no parallel orientation of the chains, it seems probable that it possesses a very well-developed system of primary valence cross-linkages which is responsible for its resistance to enzymes and chemical reagents. The presence of such a system is also shown by the behaviour of the oothecae when treated with strong swelling agents, such as concentrated solutions of lithium iodide. Lithium iodide solutions will cause substances such as silk fibroin, which is held together entirely by secondary valence linkages, to swell up, and eventually to dissolve entirely. It is, however, quite without effect on *Blatta* oothecae or on pupal exuviae of *Ephestia*, from which one may conclude that these substances owe their resistant properties to the presence of primary valence cross-linkages, which the lithium iodide is not able to disrupt.

These linkages cannot be of the same type as those of keratin, since the protein of the oothecae, and of insect cuticle in general, has a very low sulphur content. An analysis of the ootheca, after purification by digestion with pepsin and subsequent boiling in repeated changes of distilled water, gave a sulphur content of 0·15 % (equivalent to 0·56 % cystine). *Calliphora* exuviae treated in the same way gave no sulphur at all. Neither *Blatta* oothecae, pupal exuviae of *Ephestia*, nor puparial exuviae of *Calliphora* were at all softened or swollen when they were heated to boiling point in a solution of sodium sulphide at pH 10. Pieces of human finger nail used as a control were greatly swollen and eventually disintegrated completely.

NATURE OF THE SECRETION OF THE COLLETERIAL GLANDS

The material of the ootheca is derived from the colleterial glands of the female, of which there are two, one on either side, with their openings close together on the dorsal side of the vagina. Although they are morphologically left and right, they are not symmetrical; the left is much the larger of the two, and its tubules completely surround the right gland. Both of them consist of a large number of branching tubules opening into a common duct, but they differ in their histology, and in the nature of the secretions they produce. In fresh dissections the left gland appears white and slightly opalescent, and if cut across a white fluid containing crystals of calcium oxalate oozes out. The right gland is slightly more compact in structure, and it is quite transparent and colourless. The secretion is a watery solution without any crystals in it. Ito (1924) has described the histology and anatomy of the glands, and concludes that the bulk of the ootheca is formed from the secretion of the left gland, and that the right gland secretion serves to stick the eggs together at the moment of their inclusion in the ootheca. Schmalfuss and Barthmeyer (1931) observed that the oothecae contained large quantities of a dihydroxyphenol, which they suggested "might have something to do with the hardening".

The secretion of the left gland is soluble in water, in which it gives an opalescent whitish solution which is rapidly precipitated by chloroform and by all the usual protein precipitants. It gives a positive reaction with ninhydrin and with Millon's reagent, and a positive biuret and xanthoproteic test. Neither the glands when fresh, nor the lumps of coagulated secretion derived from them, will stain with black sudan B or sudan III, nor do they dissolve in fat solvents. From these tests it appears that the secretion of the left gland is a water-soluble protein, and that it does not contain any lipids in appreciable quantities.

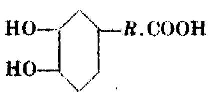
The secretion of the right gland is a clear watery fluid. The contents of the glands are not precipitated by formol, chloroform, or any of the usual fixatives, so that it appears that protein cannot be present in large amounts, although it is difficult to say that it is absent, because one cannot obtain samples of the secretion uncontaminated by proteins from the cells of the ducts. If the abdomen is opened out, and fixed in formol dichromate for 12 hr., the right gland alone gives a strongly positive chromaffin reaction, which enables it to be picked out easily from the branches of the left gland that surround it.

Lison (1936) has shown that the chromaffin reaction is a specific test for the presence of dihydroxyphenols. The colour produced is due to the production of coloured oxidation products, possibly of the type of quinhydrones, from the phenol present in the tissue. It can be shown that it is not due to the absorption of reduction products of the dichromate, since the reaction can also be performed with iodates instead of dichromates, although both the iodates and their reduction products are colourless. I have repeated the test on the right glands using potassium iodate, and find that it still gives a positive result.

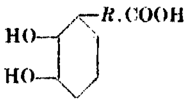
These tests afford good evidence that the right glands secrete a compound containing the catechol group, and this may be confirmed by testing the fluid from half-completed oothecae. Drops of this fluid can be obtained by pulling the oothecae out of the ventral pouch of the female before they are complete. If a drop of the fluid is tested on a slide with a dilute solution of ferric chloride, it develops a deep green colour, which changes to red on the addition of sodium carbonate; a reaction only given by ortho-dihydroxyphenols.

In the hope of being able to obtain some guide as to the precise nature of the compound secreted by the right gland, I have examined the literature for previous records of the isolation of phenolic compounds from insect material. It appears to have been generally assumed that the chromogen which acts as the precursor of melanin in insects is tyrosine or dihydroxyphenyl-alanine (dopa), but although there are numerous records of the presence of water-soluble dihydroxyphenols in insect material, I have only been able to find two instances of the isolation and identification of the supposed chromogen. Schmalfuss and Müller (1927) isolated 3-4-dihydroxyphenyl-alanine from the elytra of two species of *Melolontha*; and Schmalfuss, Heider and Winkelmann (1933) isolated 3–4-dihydroxyphenyl-acetic acid from the elytra of *Tenebrio molitor*. All the other authors who have concerned themselves with this point have been content with the extraction of various parts of the insects with water, and the demonstration that there were dihydroxyphenols present in the extract.

The secretion of the right gland is freely soluble in alcohol and ether, and cannot therefore be dopa, since dopa is very insoluble in alcohol (Schmalfuss and Werner 1926). If an aqueous extract of oothecae is shaken up with ether, some of the dihydroxyphenol present goes into solution in the ether layer, and its presence there can be demonstrated by separating off some of the ether, evaporating to dryness, and then taking up the residue in water and testing with ferric chloride. If enough sodium carbonate is added to the water to make it distinctly alkaline, it will completely remove the phenol from the ether layer. This behaviour shows that the phenol must contain a carboxy group in a side chain (Schmalfuss *et al.* 1933). From the results of the ferric chloride reaction we know that it contains two hydroxy groups in the ortho position, so that we are left with the following formula:



or



The question of whether there are any other groups attached to the benzene ring, and of the exact composition of the known side chain, can only be settled by isolating the compound and performing an analysis. Until an opportunity of doing this arises, I have assumed as a working hypothesis that the compound is 3–4-dihydroxyphenyl-acetic acid.

REACTION BETWEEN THE PRODUCTS OF THE COLLETERIAL GLANDS

If the left gland is carefully dissected out, so that there are no bits of the right gland mixed with it, it may be kept in a phosphate buffer at pH 8·0 for an indefinite time without any appreciable change in colour taking place. If the gland is dissected out and put straight into the buffer without further preparation, it generally becomes slightly brown after about a week at room temperature; but if it is washed in buffer solution after dissection, to remove all traces of blood, etc., it will keep indefinitely without becoming any darker than a pale ivory colour. I have kept preparations of left glands for 4 months in this way, using thymol or toluol as an antiseptic, without any darkening of the contents of the glands having taken place.

If left and right glands are mixed, however, and kept in a shallow dish with buffer at pH 8·0, the contents of the lumen of the left gland colour up in a way that resembles the normal darkening of the ootheca. The reaction is slow, and nothing is noticeable at room temperature until the preparation has been standing for about 12 hr., when a pale pink colour appears, particularly on those parts where some of the contents of the left gland have leaked out of the duct before denaturation set in. This colour darkens gradually to a reddish brown, which exactly resembles the colour of the ootheca or of the under parts of adult *Blatta*. After some days, the fluid in which the glands are lying may show a reddish colour, but there is never any sign of the formation of a black precipitate of melanin. Eventually, after about 10 days at room temperature, the contents of the gland become almost black, and appear as a conspicuous dark rod inside the lumen. The cells remain transparent, and only become a faint pink colour at most. The right glands by themselves never show this series of changes. If kept in buffer at pH 8·0, they may become brown after about 24 hr., but this change does not at all resemble the reaction that occurs when the two glands are mixed; the contents of the ducts are not differentiated from the cells, and the colour is a very pale khaki, as compared with a rich reddish brown or black of the left gland in mixed preparations.

A particularly striking reaction can be obtained by using the fluid secretion which surrounds the eggs inside the ootheca instead of the right glands themselves. Drops of this fluid can be obtained from fresh oothecae by pricking a small hole near one end and squeezing the ootheca gently between finger and thumb. If left glands are allowed to stand in a solution made up of this fluid diluted with phosphate buffer at pH 8·0, the material which has exuded from the cut ends of the ducts becomes bright pink after about 5 min., without there having appeared the faintest signs of colour in the solution as a whole. The colour that appears first is a slightly purplish pink, which darkens in the course of 24 hr. to a dark reddish brown, and eventually to black. This series of changes is very similar to that which occurs in the normal ootheca, as described earlier. The newly secreted ootheca is white, later becomes pink, then red, and finally dark brown or black. If an incomplete ootheca is removed from the pouch of the female and washed in distilled water, some of the white part dissolves, but such of it as remains will not darken; whereas if the ootheca is not washed, but merely kept moist with buffer at pH 8·0, the white parts darken in the normal way.

The failure of the right-gland extract to deposit a precipitate of melanin on standing confirms the view that the active principle is not an amino acid. Keilin and Hartree (1936) have found that if tyrosine is deaminated, the *p*-hydroxypyruvic acid which results is oxidized by tyrosinase, but that the reaction never gives rise to an insoluble black precipitate of melanin.

Both the darkening of the excised left gland *in vitro* and the normal darkening and hardening of the cuticle are dependent on the presence of an adequate supply of oxygen. Gortner (1911) observed that the darkening of the cuticle of newly emerged imagines of *Cicada septemdecim* could be prevented if they were kept under boiled water in stoppered bottles, and Dewitz (1916) noted that the darkening of the puparia of *Calliphora* could be prevented by covering them with water. I have confirmed Dewitz's findings on *Calliphora*, and have performed similar experiments on freshly moulted *Tenebrio molitor* larvae and imagines of *Pyrrhocoris apterus* with the same results. The reaction of the two colleterial glands is also dependent on the presence of oxygen, as is shown by the fact that if protein from the left gland is dissolved in a comparatively large volume of buffer solution (about 5 c.c.) and kept in a narrow tube, darkening starts at the surface of the liquid and spreads downwards. A more exact demonstration can be performed as follows. The glands are dissected out into phosphate buffer in the usual way, and put into a vacuum desiccator containing an alkaline solution of pyrogallol. The desiccator is then thoroughly evacuated in order to remove as much oxygen as possible from solution in the buffer, and coal gas is allowed to run in. In this way the mixed right and left glands may be kept indefinitely without any darkening taking place.

The step in this series of reactions for which the presence of oxygen is essential is presumably the oxidation of the right-gland secretion, and it is a point of some interest to determine whether or not this oxidation is catalysed by an enzyme, since at the pH at which the experiments *in vitro* have been performed, polyphenols such as catechol or dopa will oxidize spontaneously. The pH of the fluid inside the ootheca, however, as tested with B.D.H. universal indicator, seems to be about 7·0, and the reaction of the excised glands will take place at this pH, although it is slower than it is at more alkaline values.

If the cockroaches were heated to 70° C before being dissected, no reaction took place between the two glands; but this was probably due to the premature oxidation of the polyphenol, since the right glands in these preparations were discoloured, and there were yellow granules in the lumen of the ducts. More definite results were obtained by the use of sodium cyanide, which inhibits the action of polyphenol oxidase in very low concentrations (Keilin 1929). Mixed preparations of both glands remained colourless for a week if they were placed in a M/200 solution of sodium cyanide (adjusted to pH 8·0 with hydrochloric acid); while controls from the same batch of cockroaches reacted together normally, and after 4 hr. were almost black. After a week to 10 days the preparations made up with cyanide began to darken, but this may have been due to spontaneous oxidation.

When the contents of the left gland are allowed to darken *in vitro*, the resulting products share many of the properties of the mature ootheca. They are more resistant to the action of mineral acids, and to the action of pepsin and trypsin than the untreated glands, and seem to be harder when tested by pressing beneath a coverslip.

In order to show these differences to best advantage it is necessary to prepare two batches of left glands dissected at about the same time, one of which has been mixed with right glands and allowed to darken, and the other of which has been carefully freed from traces of the right gland by washing in distilled water, and, as an additional precaution, has been kept deprived of oxygen by the technique described above.

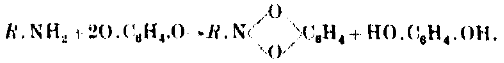
If the two samples are placed in concentrated hydrochloric acid, the controls immediately become soft and show signs of dissolution, but the darkened glands do not alter, except for a slight shrinkage. After about 36 hr. the untreated glands dissolve completely, leaving only parts of the chitinous lining of the ducts, which finally dissolve as well. The darkened glands, on the other hand, appear to be able to resist the action of the acid almost indefinitely. The differential action of pepsin is less striking, since *in vitro* the enzyme acts very slowly even on the untreated glands. Small samples of both batches are placed in dishes containing a 10% solution of pepsin in buffer (potassium chloride and hydrochloric acid) at pH 2·2, and incubated at 37° C for 3 weeks, using toluol as an antiseptic. At the end of this time the controls show almost complete solution of the contents of the lumen of the glands, and considerable corrosion of the cells; the darkened glands show some corrosion of the cells, but the contents of the ducts remain unchanged.

Similar results are given by immature oothecae when they are incubated with pepsin. The oothecae are removed from the ventral pouch of the female, cut in half, and after the eggs have been removed they are washed in ether to free them from fat. The ether is then removed by evaporation, and one half of the ootheca is placed in a pepsin solution as before and incubated at 37° C. The other half is kept in a 4% solution of formol as a control. After about a week's incubation, the white parts of the ootheca are all dissolved away, while the brown parts are only attacked in places. If the oothecae are examined under a binocular microscope it can be seen that only those parts in which the process of darkening has been incomplete, such as the white patches between the ridges along the sides of the opening, have been attacked.

The resistance of the darkened glands towards enzymes and chemical reagents suggests that, as in the mature ootheca, there is a system of primary valence cross-linkages present. The water-soluble protein secreted by the left gland cannot have any cross-linkages at all; and although on standing it coagulates and becomes insoluble in water, the coagulated protein does not acquire its resistant properties until it has darkened. The darkening is undoubtedly due to a reaction with the oxidation products of the polyphenol secreted by the right gland, and it seems reasonable to suppose that this reaction may also be responsible for the change in the chemical and physical properties which occurs at the same time.

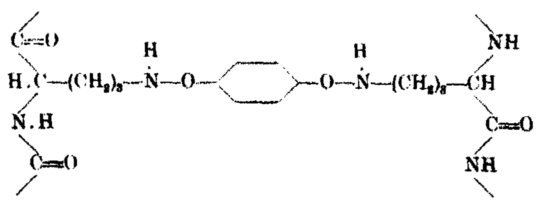
Dulière and Raper (1930) have investigated the course of the oxidation of dopa and a number of related compounds, such as methyl tyrosine and tyramine, and they find that the first product of the reaction is always an orthoquinone. This has been confirmed for catechol by Wagreich and Nelson (1938). If the initial oxidation product of the secretion of the right gland is an orthoquinone, it seems very likely that it would react with any protein present, since quinones are in general very active compounds, and a reaction between a quinone and the amino group of a protein have been described before. A reaction between the oxidation product of *p*-cresol and an amino acid was suggested by Raper (1932) as an explanation of the formation of the blue compound ("Cresol blue") which was described by Chodat (1912) as being produced when tyrosine was oxidized by tyrosinase in the presence of *p*-cresol and an amino acid such as glycine or alanine. Chodat also described reactions between the tyrosinase-*p*-cresol system and proteins, particularly partially hydrolysed gelatine. The orthoquinone derived from dihydroxyphenyl-acetic acid only differs from that derived from *p*-cresol in the possession of a carboxy group in the side chain, and it seems likely that their reactions will be similar.

Benzoquinone reacts strongly with proteins, and this reaction has been studied by chemists interested in the process of leather manufacture, since it results in the tanning of the protein. If hide powder (powdered collagen) is treated with a strong solution of benzoquinone at a pH value above 6·0, it is efficiently tanned; that is to say it no longer dissolves in hot water, and can be dried in moderate warmth without losing its characteristic texture. I have compared the effects of benzoquinone on standard hide powder with those of various vegetable tannins, such as myrobalans or oak bark, and I find that its action is very much more rapid and more efficient. The only reagent which produces a degree of heat resistance approaching that produced by benzoquinone is formol. The reaction involved in quinone tanning has been investigated by Meunier (1914) and by Thomas and Kelly (1924). Meunier suggested that the quinone reacted with the amino groups of the protein, with the formation of a quinone-collagen compound, and the reduction of one molecule of quinone to hydroquinone; and Thomas and Kelly confirmed this by showing that a high concentration of hydroquinone decreased the rate of the reaction. Meunier gives the following formula for the reaction:



$$R.NH_2 + 2O.C_6H_4.O \rightarrow R.N\left\langle\begin{matrix} O \\ O \end{matrix}\right\rangle C_6H_4 + HO.C_6H_4.OH.$$

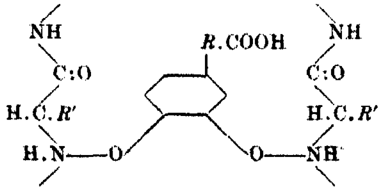
It is more in accord with modern ideas of the essential nature of the tanning process to suppose that the quinone reacts with two different amino groups attached to neighbouring protein chains, and so forms a cross-linkage between the two as in (II). The reaction may also involve the imino groups of the main chains.



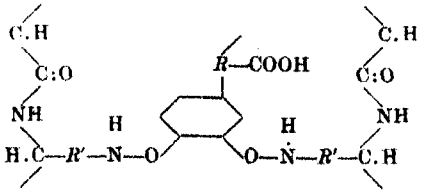
(II) Quinone tanning. Reaction with two lysine side chains.

Benzoquinone will react with the protein secreted by the left gland, and the reaction appears to be very similar to that which takes place between the protein and the right-gland secretion. By using a very dilute solution of quinone, it is possible to imitate the action of the excised right glands so perfectly that left glands treated with benzoquinone are indistinguishable from glands which have been allowed to react with the secretion of the right glands. In order to demonstrate this, the left glands are dissected out into phosphate buffer at pH 8·0 in the usual way, and a small crystal of benzoquinone is added to about 10 c.c. of the mixture. The quinone dissolves and forms a yellow solution, the colour of which soon changes to a dark pink: within 5 min. the contents of the glands begin to stain a reddish brown colour, in the same way as they do in a mixed preparation of both glands. After about a week, the protein absorbs nearly all the quinone from the solution, leaving dark brown glands in a very pale pink fluid. If the reaction is carried out in distilled water instead of in buffer, the quinone in solution remains a pale yellow, and only the protein is coloured pink, which is very like the sequence of events when the glands are treated with oothecal fluid. This behaviour is not peculiar to the proteins secreted by the colleterial glands; a similar reaction can be obtained with pieces of gelatine jelly. The properties of the glands tanned in this way are exactly those of the glands darkened by the action of the right gland secretion, and they will resist the action of concentrated hydrochloric acid in the same way. The degree of resistance which can be produced by tanning with benzoquinone does not appear to be superior to that which can be produced by allowing the natural reaction to go to completion under optimum conditions.

All this suggests that the reaction of protein with the quinone derived from the right-gland secretion is fundamentally similar to the reaction with benzoquinone, so that by analogy with the quinone reaction we may write the reaction concerned in the natural hardening of the ootheca as follows:



(I) With the imino groups of the main chains.



(II) With the amino groups of basic side chains.

In order to find out whether it really is the amino and imino groups of the protein which are concerned in the hardening reaction, I tried the effect on the reaction of first treating the glands with formol. Formol reacts with the amino groups of basic side chains of proteins (Cherbuliez and Feer 1922), and with the imino groups of the main chains (Bergmann, Jacobson and Schotte 1923). Treatment with formol should therefore occupy all the available amino and imino groups and prevent them taking part in any further reaction.

The experiment was carried out as follows. Left glands were dissected out into formol (40%) and left in the solution for 3 days. They were then washed in running water for 6 hr., and allowed to stand in dilute quinone solution, or in an extract of the right glands, for a fortnight (both solutions made up in phosphate buffer at pH 8·0). Neither of the preparations showed any reaction, and the contents of the lumen of the ducts remained perfectly white. In both experiments a small piece of untreated left gland was included as a control, and in both it darkened normally.

Treatment with formol does not affect the carboxy or carbonyl groups of the protein, so that these results confirm the supposition that it is the basic groups which are concerned in the reaction with the quinone derived from the right-gland secretion.

The vegetable tannins used in commercial practice are all complex di- or trihydroxyphenols, and there is every reason to believe that they react as such, and do not require to be oxidized to quinones. It is therefore hard to understand at first sight why the dihydroxyphenol secreted by the right gland does not react with the protein from the left gland without previous oxidation.

The reason probably lies in the differences between the types of linkage formed with proteins by various tannins. The vegetable tannins do not form primary valence links, but only electrovalent "salt linkages". According to Küntzel (1937) the phenolic hydroxy groups of the tannin form links of the type of ammonium salts with the former, and of the type of "oxonium" salts with the latter. The great firmness with which the tannin is held by the collagen is due to the large number of links involved, and to the great size and inertia of the tannin molecule, by reason of which it is less affected by thermal agitation (Lloyd and Shore 1938). The dihydroxyphenol secreted by the right gland is of comparatively low molecular weight, and so these special conditions are not fulfilled, and it does not act as an effective tannin.

Formol owes its effects to the formation of primary valence links (Küntzel 1937), and I think there is no doubt that the same is true of benzoquinone. This difference in the nature of the links formed with the protein is reflected in the difference between the effects produced by formol and benzoquinone on the one hand, and the vegetable tannins and chrome salts on the other. Benzoquinone and formol produce a degree of heat resistance, and of resistance to the action of enzymes, which is much superior to that produced by other tannins, and they react much more rapidly. Although they both combine with the basic groups of the protein, they will react at a pH well above the isoelectric point; whereas vegetable tannins will only act effectively if the solution is on the acid side of the isoelectric point of the protein, since they require maximum ionization of the basic groups.

It seems therefore that the type of reaction which occurs between the phenolic hydroxy groups of vegetable tannins and the basic groups of proteins is incapable of producing the effects observed in the "tanning" of the oothecal protein, and this explains why the oxidation of the phenol secreted by the right gland to the corresponding quinone is an essential step in the natural hardening process.

The protein of the completed ootheca is very dense and compact in texture, and this is undoubtedly an important factor in increasing its resistance to chemical reagents. A similar texture can be observed in cubes of gelatine jelly which have been thoroughly tanned with benzoquinone. After standing in a concentrated solution of benzoquinone for about 2 months, pieces of gelatine jelly shrink to about half their original size, and become as hard as wood. This change is due to the occupation of most of the strongly hydrated groups of the protein by the benzoquinone, and probably also to some extent to the polymerization of the quinone in the meshes of the protein network. The changes in the texture of the oothecal protein as it hardens are probably due to similar causes.

Küntzel (1937) has compared the process of tanning to the vulcanization of rubber with sulphur trichloride, since in both these processes the stability of a preformed molecular framework is increased by the introduction of cross-linkages. Both these industrial processes have their counterpart in nature, since the keratins of the vertebrates have justly been called "vulcanized proteins" (Astbury 1936), and the protein of the ootheca may equally well be called "tanned protein".

If the name keratin is reserved for the "vulcanized" scleroproteins which owe their stability to the presence of sulphur cross-linkages (following the definition of Goddard and Michaelis 1934), then it is convenient to have a name for scleroproteins which owe their resistant properties to the presence of aromatic cross-linkages. I therefore propose the name "sclerotin" as a general term to describe proteins such as that of the cockroach ootheca, which owe their stability to a process of natural tanning. In choosing the name, I have followed the usage of Ferris and Chamberlin (1928), who objected to the indiscriminate use of the word "chitinized" to describe the hard parts of insects, and proposed instead the non-committal word "sclerotized".

I wish to thank a great many friends for useful suggestions; in particular Dr L. E. R. Picken, who kindly took the X-ray diffraction pictures for me, and has been a constant source of helpful advice. My thanks are also due to Dr A. D. Imms, Dr C. F. A. Pantin and Professor D. Keilin for helpful criticism and advice at various stages of the work.

SUMMARY

The series of reactions concerned in the hardening and darkening of the ootheca of *Blatta orientalis* have been studied in the hope that they may throw some light on the hardening of the cuticle of insects in general. The ootheca is secreted by two glands, of which one secretes a water-soluble protein, and the other a dihydroxyphenol. After secretion the phenol is oxidized, probably by an enzyme, to the corresponding quinone, which then combines with the protein by a reaction similar to that involved in the tanning of collagen by benzoquinone. This results in the introduction of primary valence cross-linkages into the protein network, so that it becomes rigid and very resistant to most chemical reagents and enzymes. The protein of the mature ootheca is more stable than keratin, and represents a new type of scleroprotein for which the name "sclerotin" is proposed.

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On the hardening of the cuticle of insects

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[Plate 17]

In the preceding paper an account was given of the reactions involved in the hardening of the ootheca of *Blatta orientalis*. The ootheca is secreted by two glands, one of which secretes a protein, and the other an orthodihydroxyphenol. The phenol is oxidized to the corresponding quinone, which reacts with the basic groups of the protein in such a way as to introduce primary valence cross-linkages between the protein chains, and so very greatly to increase the stability of the protein. For this type of "tanned protein" the name sclerotin was proposed. In the course of the reaction the protein takes on a deep reddish brown colour, so that the process affords a close parallel to the changes which take place during the hardening of the insect cuticle in general. I have therefore investigated the hardening of various types of insect cuticle with the object of seeing whether the same mechanism is involved.

COMPOSITION OF THE EXO- AND EPICUTICLE

The most recent data on the chemical composition of the various layers of the insect cuticle are those given by Wigglesworth (1933), and summarized by him in his text-book *The principles of insect physiology* (1939). He agrees with Campbell (1929) that the epicuticle contains no chitin, and claims that it consists of a substance similar to the cutin of plants, for which he proposes the name "cuticulin". He supposes that the same substance impregnates the exocuticle of the hard parts, and that it is responsible for the characteristic amber colour of this layer.

Important conclusions as to the relationship of the amber coloured substance to the chitin of the exocuticle may be drawn from a study of the optical properties of the exocuticle. In a transverse section of the abdomen of a pupa of *Philosamia cynthia* (selected as a typical hard integument) the exocuticle is isotropic, although the endocuticle exhibits form birefringence. The isotropy of the exocuticle is not due to the absence of a suitable fine structure in the chitin, since in sections of material from which the amber coloured substance has been removed by boiling in caustic potash, the exocuticle is as strongly birefringent as the endocuticle. The absence of birefringence must then be due to the fact that the intermicellar spaces of the chitin are completely filled by a material of refractive index about 1·54 or 1·70 (see Schmidt 1934).

The relations between the amber coloured substance and the chitin is thus seen to be very like that between the cellulose and the lignin in lignified plant tissue, as described by Freudenberg, Zocher and Dürr (1929). This comparison was first made by Schulze in 1922, and formed the basis of his theory of the chemical nature of the substances responsible for the hardening of the insect cuticle.

THE "INKRUSTEN"

Wigglesworth does not produce any original hypothesis as to the nature of the substances responsible for the hardness of the cuticle. He remarks that they are removed by the same treatment as removes the melanin and cuticulin from the exocuticle, and for the rest he accepts the term "Inkrusten" coined by Schulze (1922). He quotes Schulze and Kühnelt as having produced some evidence of their being carbohydrates. The idea of the "Inkrusten" is mainly due to Kühnelt (1929). He regarded them as impregnating the chitin of the exocuticle as lignin impregnates cellulose in wood, and he suggested that they might be in some form of loose chemical combination with the chitin. His evidence for the statement that the "Inkrusten" are carbohydrates consists of two observations. The first is that small pieces of exocuticle when dissolved in concentrated hydrochloric acid "as far as possible without warming", will give a purple colour with an alcoholic solution of α-naphthol, thus showing the presence of furfurals. The second is that pure chitin will dissolve in concentrated acids without the formation of dark "humin substances", whereas bits of exocuticle form an abundant dark precipitate. With regard to the first of these observations; chitin is very easily hydrolysed by strong acids, and the glycosamine so formed would give a positive reaction with α-naphthol. The second test might be given by almost any impurity in the chitin. Thus we see that the chemical evidence in favour of Kühnelt's hypothesis is very weak, and the term "Inkrusten" is perhaps better dropped altogether.

THE EPICUTICLE. NATURE OF CUTICULIN

Although Kühnelt (1928) claimed that the epicuticle could be stained with sudan III, Wigglesworth states that cuticulin is not stained by any fat stains. I have been unable to confirm Kühnelt's statement, but I find that the epicuticle of larvae of *Calliphora* is stained very strongly by the new fat stain black sudan B, introduced by Lison (1936). This test is particularly strong in larvae of *Calliphora*, and is even given by sections cut in paraffin. It is also given, although less strongly, by the epicuticle in paraffin sections of the pupa of *Philosamia cynthia*; but not in paraffin sections of *Rhodnius prolixus*, *Ranatra linearis*, or *Locusta danica*. In *Locusta* and *Rhodnius* the test is not positive even in sections cut with the freezing microtome, which suggests that if there is lipid present it must be masked in some way. The colour given by the epicuticle of *Calliphora* is like that given by the cuticle in paraffin sections of plant material, but is not quite so brilliant.

When the larval cuticle of *Calliphora* is converted into the puparium, there is a great increase in the amount of the amber coloured substance present, but the extent of the layer which stains with black sudan B remains the same. The amber coloured part of the cuticle of *Philosamia*, or of *Rhodnius*, does not stain. This suggests that the amber coloured material in the exocuticle does not contain lipids, and that the colour is not due to chromolipoids, since, according to Lison, chromolipoids do not cease to stain with fat stains even after they have reached a degree of polymerization such that they are completely insoluble in fat solvents. A careful repetition of the tests on which Wigglesworth based his theory of the composition of cuticulin confirmed these conclusions.

When exuviae of *Rhodnius* were gently heated on a slide in dilute nitric acid, there was some evolution of gas, and the amber coloured parts melted into a viscous yellow fluid. Underneath this fluid the epicuticle was still visible, with some of the bristles still intact. In places the yellow fluid ran off the epicuticular layer, which then remained as a perfectly transparent membrane, with no apparent colour of its own. The same behaviour can be seen in frozen sections, in which the amber coloured part of the exocuticle melts and leaves a thin colourless strip of membrane, which is evidently the epicuticle. The behaviour in Schulze's medium is the same; the whole melts, leaving a transparent membrane, which on further heating melts into droplets, which stain with black sudan B and sudan III. These drops are soluble in xylol if slightly warmed, but always leave a small residue. If the melting is stopped at a stage at which the epicuticle is still intact, the whole membrane that is left will stain with black sudan B. The yellow drops which are obtained from the initial melting do not stain with fat stains. By cooling they can be obtained as solid lumps, which are stiff and brittle, and are not soluble in water, dilute acids, benzene, alcohol, or ether, but will dissolve in dilute alkalies to give a deep orange solution. If the exuviae, or frozen sections of the cuticle, are heated with Millon's reagent (prepared according to Bensley and Gersch 1933) the yellow substance first of all gives a strong positive reaction, gradually loses its colour as the heating is continued, and finally melts into a yellow mass similar to that obtained when dilute nitric acid alone is used.

These reactions, combined with the results of the staining experiments, show that the lipids of the cuticle are almost entirely confined to the epicuticle, even where the exocuticle is hard and yellow. From its reactions with Millon's reagent it appears that the amber coloured material contains aromatic groups.

To confirm the presence of aromatic compounds, the argentaffin test (Lison 1936) has been used. Tested by this method, all hard exocuticles give a strongly positive result. I have tested sections of puparia of *Calliphora erythrocephala*, pupae of *Ephestia kuehniella*, *Aporia crataegi*, and *Pieris brassicae*, and elytra of *Tenebrio molitor* and *T. obscurus*, and in all of them the test is positive. There can be no doubt that the results are due to the presence of polyphenols and not to any other reducing agents, since Schmalfuss and Barthmeyer (1931), after testing a great number of species belonging to different orders, found that if the cuticle was ground up and extracted with hot water it was nearly always possible to demonstrate the presence of ortho-dihydroxyphenols by the ferric chloride reaction. This I have been able to confirm on all the species tested by the argentaffin test.

The argentaffin test is also positive in the epicuticle of soft insects, such as the larvae of *Calliphora*. If the exocuticle is not pigmented, the epicuticle alone reacts. This is true for the larvae of *Calliphora* and *Ephestia*, and for the intersegmental membranes of larvae of *Tenebrio*.

A more specific test for phenols is the "diazo reaction" described by Lison (1931). This test suffers from the objection that the dyes formed in a positive reaction are nearly always yellow or orange, so that they are difficult to distinguish from the natural colour of the exocuticle. Lison gives various ways of overcoming this difficulty, but in these more elaborate procedures it is impossible to avoid the formation of coloured by-products, which act as dyes, and so obscure the results. By using the di-diazo of dianisidine sulphonic acid, and introducing α-naphthol into the molecule, I succeeded in obtaining a reddish purple coloration of the epicuticle of *Calliphora* larvae, which may be taken as to some extent confirming the results of the argentaffin test.

These reactions of the epicuticle indicate that although it does contain highly polymerized and insoluble lipids of the type of cuticulin, it also contains dihydroxyphenols. Since it is very probable that in the early stages it also contains proteins, it seems reasonable to assume that it is composed mainly of sclerotin, and that it is secondarily impregnated with lipids, which by a process of condensation and polymerization may become insoluble in fat solvents.

According to Wigglesworth (1933), the epicuticle is secreted as a hydrophil membrane which is freely permeable to water, and stains strongly with basic dyes. This membrane in fact has all the properties of a simple protein. I have not tested newly secreted epicuticles from *Rhodnius*, but the epicuticle of the elytron of a *Tenebrio molitor* pupa, taken from an individual which had not yet begun to darken, stained with iron haematoxylin and did not give a positive argentaffin test. Evidently the epicuticle is secreted as a simple protein, and is only later "tanned" and impregnated with lipids. The source of the lipids has not been determined, but they are probably derived from glands opening on to the surface of the body. The process of "tanning" will facilitate subsequent impregnation with lipids, since most of the strongly hydrated groups of the protein are involved in the reaction with the tanning agent.

Apart from this effect of suppressing hydrated groups, the addition of a large proportion of aromatic groups is calculated to make the material more lipophil (Mark 1938). Gelatine which has been thoroughly tanned with benzoquinone can be impregnated with oils without being dried first, if superficial water is removed with filter paper and the material is dipped in paraffin or olive oil. Once oiled in this way, the gelatine remains strongly hydrofuge, and the oil cannot be removed by rinsing in water, whereas if dried gelatine which has not been previously tanned is soaked in paraffin, the oil can be removed by washing in a stream of water, so that the surface becomes hydrophil again. *Blatta* oothecae undergo a progressive change in the ease with which they can be impregnated with lipids as darkening proceeds. If a partially formed ootheca which is still white at one end, is dipped in paraffin, the oil spreads over the surface of all the brown part, but stops short at the boundary of the white part. Fairly fresh oothecae removed from the female's pouch are not necessarily hydrofuge, even when they are completely darkened, but the surface of old oothecae always is: it seems probable that an oily secretion from the cuticle of the mother spreads over the surface of the ootheca during the period in which it is carried in the ventral pouch. I have noticed that when a female with a ripe ootheca is cleaning herself with her hind legs she usually includes the projecting part of the ootheca in her attentions.

The epicuticle of some insects remains hydrophil throughout life, and so cannot contain any considerable quantities of lipids. The epicuticle of *Psilopa petrolei* (Diptera, Ephydridae) described by Thorpe (1930) is an example of this type. The larvae live in pools of crude petroleum, and breathe through their posterior spiracles, which are supported at the surface by a ring of fringed valves which are not wetted by the oil. Through the kindness of Dr Thorpe I have been able to examine sections of the larvae, and I find that the epicuticle is in most respects very like that of *Calliphora* larvae, and that there can be no doubt that it is a true epicuticle.

In *Peripatopsis* (Onychophora) both hydrophil and hydrofuge types of epicuticle occur in different parts of the body. The integument of *Peripatopsis* is thin and soft, and there is a well developed epicuticle, which stains with iron haematoxylin in the same way as the epicuticle of larvae of *Calliphora*. The general body surface is hydrofuge as in insects, but in the region of the mouth the epicuticle is hydrophil, and in life is kept moist by the saliva (personal communication by Dr Manton). In some paraffin sections of *Peripatopsis capensis* which Dr S. M. Manton kindly gave to me, I applied the argentaffin test, and found it positive, both over the general body surface, and in the region of the mouth. The epicuticle in these sections did not stain with black sudan B. In the mouth region the epicuticle is slightly thicker and less papillose than over the rest of the body, but there is no other difference.

A reduction in the extent to which the epicuticular proteins are "tanned" is probably one of the factors responsible for the hydrophil nature of these epicuticles. The epicuticle of *Psilopa* gives a positive argentaffin reaction, but not so strongly as that of *Calliphora* larvae. The spines on the cuticle of *Psilopa* are remarkable in that they stain all through with iron haematoxylin: this is quite unlike the behaviour of similar spines in *Calliphora* larvae, which only stain at their bases, and at their tips are hard and yellow (figures 2, 3). This suggests that the epicuticular proteins of *Psilopa* still retain a fairly high proportion of free reactive groups; a view which is supported by the observation of Thorpe that if the larvae are removed from the oil they soon dry up and die, presumably because their cuticle is excessively permeable to water.

HOMOLOGY OF THE EPICUTICLE WITH THE CUTICLE OF CRUSTACEA

The hypothesis outlined above has the advantage of making possible some attempt to homologize the epicuticle of insects with the cuticle of Crustacea. Wigglesworth (1933), impressed by the difference between the method of formation of the crustacean cuticle as described by Yonge (1932), and that of the insect epicuticle, has emphasized the differences between the two; but a series of comparative tests shows that chemically at any rate, they have much in common. For these tests I have used sections of decalcified lobster integument and small pieces cut off the cuticular pads of the gastric mill. The cuticle resembles insect epicuticle in its resistance to the action of acids, and gives a strongly positive xanthoproteic and Millon's reaction, showing that it must contain aromatic groups. In sections the argentaffin test is positive, but much more feeble than in insect material: the extent to which the cuticle reduces ammoniacal silver oxide varies from one part of the body to another. Like the insect epicuticle, the crustacean cuticle is isotropic. The great thickness of the cuticle in the Decapoda studied by Yonge is rather unusual; in other groups (e.g. the Peracarida), the cuticle is about the same thickness as the epicuticle of insects, and in sections stained with iron haematoxylin the similarity is very striking.

The most important difference between the insect epicuticle and the crustacean cuticle concerns the method of formation. According to Yonge, the crustacean cuticle is secreted by the epidermal glands after the secretion of the chitinous layers has begun; whereas in insects the epicuticle is secreted by the cells of the hypodermis before they begin to secrete the chitin. Yonge's views have not been confirmed by Drach (1939), who states that in *Maia squinado* the cuticle is formed in the same way as the epicuticle of *Rhodnius* described by Wigglesworth (1933).

The other points of difference that Wigglesworth emphasizes are that the cuticle of the Decapoda stains with basic dyes and fat stains, and that it imbibes water. The difference in the ease with which the lipids will take up fat stains is probably due to the fact that the lipids of the crustacean cuticle are much less oxidized and polymerized than those of the insect epicuticle. The other points may all be explained as being due to the fact that the crustacean cuticle is much less thoroughly tanned than the insect epicuticle, and so has more active groups free to take up water and stains.

THE EXOCUTICLE

The evidence so far discussed strongly suggests that the amber coloured material of the exocuticle is closely similar to the sclerotin of cockroach oothecae. To confirm this I have made a study of the actual process of hardening in various types, in order to investigate the precursors of the amber coloured substance.

THE HARDENING OF THE PUPA OF *EPHESTIA*

The first subject in which the hardening of the cuticle was studied was the typical amber coloured pupa of the moth *Ephestia kuehniella*. The process of pupation has been described by Kühn and Piepho (1938), and the following account is largely taken from their paper. Immediately after ecdysis the cuticle is thin and transparent, so that the green blood of the pupa shows through ("green pupa stage"). The exocuticle at this stage is strongly stained by iron haematoxylin. The hardening and darkening of the exocuticle begins soon after ecdysis, and is more or less contemporaneous with the secretion of the endocuticle. As the exocuticle darkens, it loses its staining properties, and gradually acquires its final clear yellow colour. The process of darkening is evidently accompanied by the secretion of a polyphenol into the new cuticle, since the exocuticle of the green pupa stage will not reduce ammoniacal silver oxide, although the amber coloured exocuticle does so strongly.

More information than can be obtained by studying the natural processes can be had from a study of the abnormal process of "partial pupation" described by Kühn and Piepho (1938). This is produced by ligating off the head of the larva at about the critical period, when the hormone that causes pupation is just being secreted into the blood. It occurs in several different forms, differing in the degree to which they resemble normal pupation. The least severe form is that in which the hypodermis secretes a new pupal cuticle without the appearance of an exuvial space, so that the new cuticle sticks tightly to the old larval skin. In specimens showing a more intense form of partial pupation, the new cuticle begins to harden and darken, but since there is no exuvial space, some of the materials which are responsible for the darkening and hardening pass right through the new cuticle, and diffuse into the old larval cuticle, which takes on the appearance and consistency of a normal pupal cuticle. According to Kühn and Piepho, the old larval cuticle first swells up and begins to stain very strongly with iron haematoxylin (which it does not normally do), and then begins to darken and harden like the cuticle of a normal pupa, and loses its staining properties in the same way. A slightly stronger development of the partial pupation results in the appearance of a small exuvial space filled with fluid in the middle of the affected area. When an exuvial space is formed in this way, the hardening materials diffuse out of the new cuticle and become precipitated around the edges, so that in sections they appear as small round concretions in the narrow crack separating the old and the new cuticle. These concretions can be found in all stages of hardening and darkening: some stain deeply with iron haematoxylin, while some are already yellow and transparent. I have repeated these experiments, and have obtained partially pupated larvae, which were fixed in aqueous Bouin, and sectioned in paraffin. The concretions in the exuvial space reduce ammoniacal silver oxide strongly (figure 1), and do not stain with black sudan B. They also give a positive result with Lison's dianisidine sulphonic acid di-diazo method (using α-naphthol), although this cannot be regarded as at all conclusive, since they would certainly stain with the various by-products which are produced.

These results indicate that, at the time of browning and hardening, substances are added to the chitinous cuticle in a fluid form, which later become hard and non-reactive, and that among these substances are dihydroxyphenols.

FORMATION OF THE PUPARIUM IN *CALLIPHORA*

The second subject in which I studied the process of darkening and hardening was the puparium of the blow-fly *Calliphora erythrocephala*. In *Calliphora*, as in all the higher Diptera, the process of pupation does not involve the secretion of a completely new cuticle, but only the hardening of the cuticle of the last larval instar, which is impregnated with amber-coloured material, and becomes the puparium.

I fixed a series of puparia, at various stages of the process of darkening and hardening, in formol, and cut sections with a freezing microtome. Very little difference could be detected between the various stages by the use of Millon's reagent. This I took to be due to the loss of the phenolic component from the sections as they were floating in water after being cut, since it is very difficult to fix phenols unless they are combined with a protein (Lison 1936). Using a similar series, unfixed, I tried the effects of ninhydrin (triketohydrindene hydrate), and found that if the sections were transferred as quickly as possible from water to the slide after cutting, very good results were obtained. The stages in which browning was just about to begin gave a strongly positive result in the cuticle, showing the presence of amino acids (or conceivably of ammonium salts, see Shore and Lloyd 1938). Larvae which had not begun to pupate only gave a very faint reaction.

Other material, fixed in Bouin and cut as paraffin sections, gave histological results very similar to those obtained by Kühn and Piepho on *Ephestia*. The exocuticle of the larvae does not normally stain with iron haematoxylin, or give a positive argentaffin reaction, although the epicuticle does both. The mature brown puparium stains strongly with haematoxylin when fresh, but the older examples begin to lose their staining reactions in the outer layers. The brown parts of the puparium always give a strongly positive argentaffin reaction (figure 4). It is difficult to obtain satisfactory sections of the intermediate stages from normal larvae, and I therefore tried to induce some form of "partial pupation" in this material as well as in *Ephestia*.

The method used was to burn out Weissman's ring with a small electric cautery as described by Burtt (1938). About 200 larvae, from a batch that was ready to pupate, were operated upon in this way. Most of them did not pupate at all, but among those that did there were several in which hardening and darkening of the cuticle did not take place evenly all over the body, and many in which hardening took place without any of the usual changes in shape. These latter animals preserved the conical shape of the larva, but became hard and brown, thus showing that the hardening does not depend on the change in the shape of the body. In those that did not pupate evenly all over, sections showed very well the sequence of changes that take place in the cuticle preparatory to hardening. These are exactly as described in *Ephestia*; in the initial stages the cuticle stains very strongly with iron haematoxylin, and later becomes hard and yellow and loses its staining properties. The results of the tests with ninhydrin make it likely that the substance in the cuticle which is responsible for the staining with iron haematoxylin is a protein; and this is confirmed by the results of Evans (1932), who found that the amino acid nitrogen in the blood of *Lucilia* larvae fell steeply at the time of hardening of the cuticle, while the skeletal nitrogen (defined as the nitrogen remaining in the skeleton after hydrolysis with 1% caustic potash for 4 hr.) rose. In two later papers Evans has published the results of similar analyses of *Tenebrio molitor* (1934) and *Vespula germanica* (1938), in both of which he obtains similar results.

GENERAL CONCLUSIONS

The investigations of the hardening of the cuticle in *Ephestia* and *Calliphora* have shown that there are present at the time of hardening and darkening of the cuticle a protein and a dihydroxyphenol. It is well established that the presence of oxygen is essential for normal hardening and darkening to take place; and the finished amber coloured substance in the exocuticle of hard insects has all the properties of the sclerotin of cockroach oothecae. From these data it seems justifiable to conclude that the amber coloured substance in the exocuticle is a form of sclerotin. From the histological evidence it appears that the protein and the phenol, which are both water soluble compounds, are secreted into the cuticle soon after ecdysis, probably by way of the pore canals, and that they react *in situ*.

Further evidence of the similarity of the amber coloured material of the exocuticle and the sclerotin of the oothecae of *Blatta* can be obtained from a comparison of their refractive indices, and of their nitrogen content. The refractive index of the ootheca was determined by immersing sections in fluids of different refractive index, and observing the movements of Becke's line on focussing up and down. The refractive index is almost exactly equal to that of nitrobenzene (1·553), and is above that of ethylene dibromide (1·539), but below that of benzyl benzoate (1·570) (the fluids used were all B.D.H. "R.I." reagents). This figure is in fairly good agreement with the value of 1·54 arrived at for the amber coloured substance from a consideration of the optical properties of the exocuticle.

The figures for the percentage nitrogen in the exocuticle were obtained from puparial exuviae of *Calliphora erythrocephala*. They were arrived at by taking the nitrogen content of the entire exuviae and then making a correction for the chitin present. The figure for the chitin nitrogen was determined from exuviae which had been heated at 120° C for 12 hr. in a concentrated solution of caustic potash to remove all the sclerotin: in order that the figure so obtained shall apply to the original chitin, however, an allowance must be made for the de-acetylation of the chitin during the potash treatment, and this introduces a considerable error, so that the figures given are only approximate (table 1).

TABLE 1

Blatta ootheca		*Calliphora* puparium		
Sulphated ash	14·9 %	Total nitrogen	10·3 %	
Ash reckoned as oxalate	14·1 %	Chitin nitrogen	3 %	Approx.
Nitrogen corrected for ash	12·5 %	Sclerotin nitrogen	13 %	

The nitrogen content of both the exocuticular sclerotin and that of the ootheca is rather low for a protein, but it is such as might be expected after the addition of a high proportion of a non-nitrogenous phenol. The values shown in table 1 are not unlike those given by Aronssohn (1910) for the composition of an unknown substance from bee larvae. There is also some similarity with the "pupine" of Griffiths (1892), and with the "uranidins" of Krukenberg (1884). The descriptions of these substances are not sufficiently exact, however, for it to be worth while to compare them in detail.

If the hardening of the cuticle of insects is due to the formation of sclerotin, it is to be expected that the phenolic substance in the blood, which is responsible for the blackening of the blood after death or at the site of injuries, should be in some way concerned in the process. The oxidation products of the phenol in the blood will not react with proteins however. If the left colleterial gland from a female *Blatta* is placed in a solution of cockroach blood diluted with phosphate buffer at pH 8·0, no change occurs in the colour of the gland. Instead, there is a copious precipitate of melanin, which settles on the bottom of the dish. The same thing happens if, instead of diluted blood, a 0·1% solution of dopa in buffer is used. Unlike the phenol secreted by the right colleterial gland of *Blatta* (see the first paper of this series), the phenol in the blood is not soluble in alcohol; if cockroach blood is mixed with an equal volume of alcohol it gives a precipitate, and the clear fluid remaining, if decanted off, does not give a positive reaction with ferric chloride. From this it appears that the phenol in the blood is not identical with the secretion of the right colleterial gland of *Blatta*: it seems probable that it really is dopa, as it has often been assumed to be in the past.

Since the phenol in the blood is not capable of reacting with protein *in vitro*, it does not seem likely that it is directly concerned in the formation of sclerotin. The best theory to account for its presence in the blood seems to be that it is being transported from some organ in which it is synthesized to the cells of the hypodermis. Such a theory agrees well with the observations of Millot and Jonnart (1926) on spiders. These authors found that in the blood of *Araneus diadematus* and of other spiders, there were cells containing granules of a dihydroxyphenol, which were numerous just before ecdysis, but disappeared altogether after ecdysis and the hardening of the new cuticle. Henke (1924) has recorded similar fluctuations in the chromogen content of the blood of the plant bug *Pyrrhocoris apterus*.

The compound directly concerned in the formation of the sclerotin in the cuticle is probably dihydroxyphenyl-acetic acid, or some closely related compound. This substance has been isolated from the elytra of *Tenebrio molitor* by Schmalfuss, Heider and Winkelmann (1933), and in the first paper of this series reasons were given for supposing that this is the phenol concerned in the formation of sclerotin in the ootheca of the cockroach. Such a compound should be soluble in alcohol: the only record of alcohol-soluble phenolic compounds from insects is that of Villon (reported by Slater 1887) who isolated an alcohol-soluble "tannin" from *Calandra granaria*. It appears, however, that alcohol-soluble dihydroxyphenols are widely distributed in insects, since in some preliminary tests I was able to demonstrate their presence in dried museum specimens of the following species (table 2).

TABLE 2

Geotrupes stercorarius (Coleoptera)	*Dicranura vinula* (Lepidoptera)
Anoplocnemis tristator (Hemiptera)	*Aeschna cyanea* (Odonata)
Bombus lapidarius (Hymenoptera)	*Eristalis tenax* (Diptera)

The method used was to grind the specimens finely with sand, and to extract the powder with hot alcohol. The alcohol was then filtered, evaporated to dryness on a water bath, and the residue taken up in distilled water and tested with ferric chloride and sodium carbonate.

If the polyphenol concerned in the formation of sclerotin is transported in the blood in the form of an amino acid, it must be deaminated in the hypodermal cells. The deamination of dopa in these cells would account for the presence there of concretions of uric acid, as observed by Wigglesworth (1933) in *Rhodnius*. The uric acid granules in *Rhodnius* are confined to the cells underlying the sclerotized placques at the bases of the bristles, and Wigglesworth explains their distribution as being due to the fact that the parts of the hypodermis in which they occur are "sheltered from the blood", so that the elimination of waste products is difficult. However, if the deamination of an amino acid from the blood is an essential preliminary to the formation of sclerotin, the accumulation of nitrogenous waste products in parts of the hypodermis where sclerotin formation is particularly active needs no further explanation.

I am indebted to numerous colleagues, particularly to Mr E. T. Burtt, Dr W. H. Thorpe and Mr D. A. Webb, for help and advice during these investigations.

SUMMARY

The hardening of the insect cuticle is due to the formation of a protein similar to that of the cockroach ootheca. A water-soluble protein and a dihydroxyphenol are secreted into the outer layers of the cuticle, and oxidation products of the phenol there react with the protein to form sclerotin. A similar reaction takes place in the epicuticle, which is secreted as a protein membrane and is subsequently "tanned" by the introduction of aromatic cross-linkages. After it has been converted to sclerotin in this way, the epicuticle is impregnated with lipids, which oxidize and polymerize until they become insoluble in fat solvents. The cuticle of the Crustacea is like the epicuticle of insects in chemical composition, but differs in that the lipids are less highly polymerized.

The phenol which takes part in the reaction to form sclerotin is probably dihydroxyphenyl-acetic acid, or some very similar compound: the phenol in the blood, which is responsible for melanosis after death or at the site of injuries, will not react with protein *in vitro*, and so cannot be directly concerned in the formation of sclerotin. The blood phenol appears to be dihydroxyphenyl-alanine, which is transported in the blood from some organ where it is synthesized to the cells of the hypodermis, where it is deaminated, and converted to the phenolic component of sclerotin.

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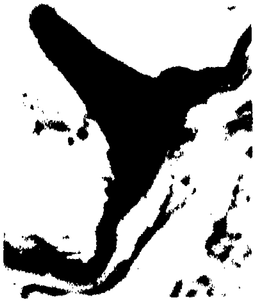

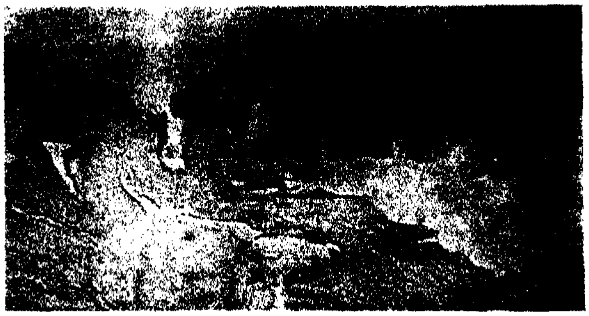

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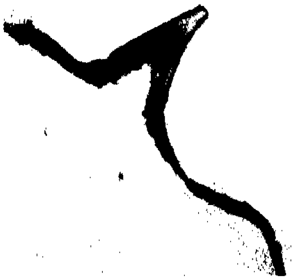

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FIGURE 1. Transverse section through dorsal wall of abdomen of larva of *Ephestia kuehniella*, showing a partially pupated area. Treated for 24 hr. with 10 % ammoniacal silver oxide. (× 300.)

FIGURE 2. *Psilopa petrolei*. Section of larval cuticle to show spine. Iron haematoxylin. Note that the spine has stained right up to the tip. (× 250.)

FIGURE 3. *Calliphora erythrocephala*. Section of larval cuticle to show spine. Iron haematoxylin. Note that the distal part of the spine has not stained. (× 250.)

FIGURE 4. *Calliphora erythrocephala*. Transverse section of larval cuticle. Ammoniacal silver oxide for 24 hr. Note that only the epicuticle has reduced the solution. (× 100.)

FIGURE 5. *Calliphora erythrocephala*. Transverse section of fresh puparium. Ammoniacal silver oxide for 24 hr. Note the heavy reduction in both exocuticle and epicuticle. (× 100.)

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The effect of unilateral castration on spermatogenesis

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INTRODUCTION

Many factors influencing spermatogenesis and fertility in the male have been studied; these include seasonal changes, hypophysectomy, hormones and vitamins, and the plan of experiment has usually consisted of treatment followed by a qualitative histological examination of the testis. In the female the effect on fertility of a given treatment may be measured accurately and quantitatively by counting the number of large follicles, the number of corpora lutea or the number of young conceived. An early attempt to apply quantitative standards to male fertility was made by Lloyd-Jones and Hays (1918). In studying the effect of frequent copulations on the volume and density of the semen, ejaculates of male rabbits were withdrawn from the vaginas of the living females; the laborious nature of the technique and the lack of certainty that all of each ejaculate could be recovered in this way may be imagined.

The application of accurate quantitative standards has had to await the development of a suitable technique for collecting ejaculates; such a method has now been evolved to meet the requirements of artificial insemination. Collection of semen by use of an artificial vagina is the standard technique employed for this purpose, and it may be used either in conjunction with a female or an artificial female. The latter may be constructed so as to resemble the female of the species concerned, or, as in the case of the rabbit, may simply take the form of a cured pelt laid over the arm of the operator. The collecting technique has been fully described by Macirone and Walton (1938).

This technique has been used in the present experiment, the first of a series of studies of factors influencing fertility in the male, and, more particularly, factors influencing the production of spermatozoa.

MATERIAL AND METHODS

The rabbit is a very suitable animal for a study of this nature; it can be readily trained to the collecting technique, and because of the very characteristic orgasm that accompanies ejaculation there is no doubt, such as sometimes exists in the case of males of some other species, that ejaculation has actually taken place.

Eight young bucks of a commercial strain were used in the experiment. They were fed on a ration of crushed oats and bran, mangels and sanfoin hay. Growth curves were kept by means of weekly weighings.

Ten ejaculates were collected from each buck on one day in each week. The ejaculates were taken in pairs at intervals of 2 hr., the first pair at 9 a.m. and the last at 5 p.m. There was an interval of about 5 min. between the first and second ejaculate of each pair. [Previous experience (Edwards and Walton 1939) had shown that an exhaustion test of this kind is superior to isolated collections where reserves and rates of production of spermatozoa are to be measured.] Before the ninth collection a doe in oestrum in which the Fallopian tubes had been cut was introduced to the cage of the buck and attempts at copulation were permitted. The doe was then withdrawn and the ninth collection taken. This was done to prevent, as far as possible, any reduction in the ejaculation of spermatozoa that might be caused by the gradual satiation of sexual appetite as stimulated by the artificial female, and although it soon became apparent that this factor was of little significance the practice was continued until the end of the experiment. It was also adopted on the few occasions—27 in 1760 collections—on which a buck seemed to require the stimulus.

After each group of paired collections had been made the volumes of semen were measured. The jelly-like coagulum, secreted by the uterus masculinus, usually present in the first and second ejaculate though very rarely occurring in subsequent ejaculates, was first removed. The density of the spermatozoa in the ejaculate was then measured by the technique described by Walton (1927).

It was found that about 85 % of the total number of spermatozoa produced in the ten ejaculates in one day were present in the first six. Because ejaculates 7, 8, 9 and 10 and, more particularly, ejaculates 8 and 10 were of extremely small volume it was decided to adopt the total number of spermatozoa in the first six ejaculates as the index of the rate of weekly production.

THE EFFECT OF UNILATERAL CASTRATION

The four experimental bucks were selected after five weekly collections had been made. None of the bucks had experienced an ejaculation before the first week of the experiment, and it was necessary to remove accumulated spermatozoa before pairing them on a basis of rate of sperm production. In table 1 it will be seen that this was achieved after the second week, and that from the third to the fifth week the average rate of production in the group of bucks subsequently to be unilaterally castrated bore a constant relation to that of the control bucks. Immediately after the tenth collections on the fifth week the left testis in bucks 16 and 11 and the right testis in bucks 8 and 1 were removed under ether anaesthesia.

The unilateral castration did not affect the sexual vigour of these bucks either immediately afterwards or at any subsequent stage of the experiment. Collections were made for a further period of 19 weeks; after the tenth and seventeenth weeks an interval of 14 days was allowed in order to observe the effect of the longer interval.

In table 1 results relating to 1056 ejaculates are summarized. In all except three cases the weekly total number of spermatozoa is that contained in the first six ejaculates. In three cases perfectly normal copulations, as indicated by the orgasm, were not accompanied by ejaculation of semen and later collections were included to complete the group of six.

It is apparent from these results (arranged for control and experimental group averages in figure 1) that unilateral castration did not result in

TABLE I. NUMBERS OF SPERMATOZOA (IN MILLIONS) PRODUCED BY CONTROL AND EXPERIMENTAL BUCKS IN A TOTAL OF SIX EJACULATIONS COLLECTED AT INTERVALS OF 1 WEEK

No. of coll.	Buck 9 control	Buck 8 exp.	Buck 3 control	Buck 1 exp.	Buck 13 control	Buck 16 exp.	Buck 10 control	Buck 11 exp.	Average control	Average exp.	Exp. group as % of control
1	898	967	544	719	470	466	250	268	543	605	111·4
2	429	429	258	390	285	194	177	262	287	319	111·1
3	484	424	263	234	282	254	149	117	294	257	87·4
4	407	405	300	212	224	165	202	207	283	248	87·6
5	450	357*	207	317*	288	207*	199	110*	286	248*	86·7*
6	469	164	294	131	223	169	226	47	303	127	41·9
7	465	238	300	161	337	135	206	76	327	152	46·5
8	485	243	291	174	320	207	179	62	319	171	53·6
9	594	138	305	139	316	167	277	97	373	135	36·2
10	606	239	292	195	304	189	264	107	367	182	49·6
11											
12	935	518	748	408	641	320	512	255	709	375	52·9
13	544	293	333	242	378	317	180	185	359	259	72·1
14	516	284	394	208	454	192	193	141	389	206	52·9
15	664	326	350	195	471	167	268	143	438	208	47·5
16	466	240	356	182	426	161	236	127	371	177	47·7
17	616	305	411	182	504	180	280	120	453	197	43·5
18											
19	1018	527	646	567	759	400	384	318	702	453	64·5
20	545	499	432	199	339	187	203	148	380	258	67·9
21	598	261	371	307	468	172	254	138	423	219	51·7
22	795	274	308	260	533	146	226	153	466	208	44·6
23	491	309	432	220	496	194	220	145	410	210	52·9
24	704	312	344	290	519	182	229	160	449	236	52·5
Total no. of ejaculations	132	132	132	132	132	132	132	132	528	528	

* One testis removed after collection made on this day. (No collections were made at the 11th and 18th weeks.)

hypertrophy of function in the remaining testis; the rate of spermatozoa production over the period of 19 weeks following operation remained at approximately one-half of that of the control group. As the volume of the ejaculate was not affected (table 2) the effect was seen in a reduced density of spermatozoa. This finding is in contrast with that described by Smelser (1933) of the result of unilateral castration in the rat. There is, however, an important difference in technique which may explain the difference in result; spermatogenic activity in the remaining testis was measured, not, as in the present case, by regular collections of semen, but by counting the number of spermatozoa present in the epididymis 14–68 days after operation. In the single epididymis spermatozoa were found equal in number to those recovered from two epididymes in the control animals. It is possible that as a result of unilateral castration and in the absence of regular withdrawal of spermatozoa from the epididymis a greater accumulation occurred in the experimental animals. It is also to be noted that the weight of the testis, which is a good indicator of spermatogenic activity (p. 417), is not correlated with the weight of the epididymis (p. 413), and it is therefore not certain that the numbers of spermatozoa found in the epididymis are a measure of rate of production. There is also the possibility of a species difference.

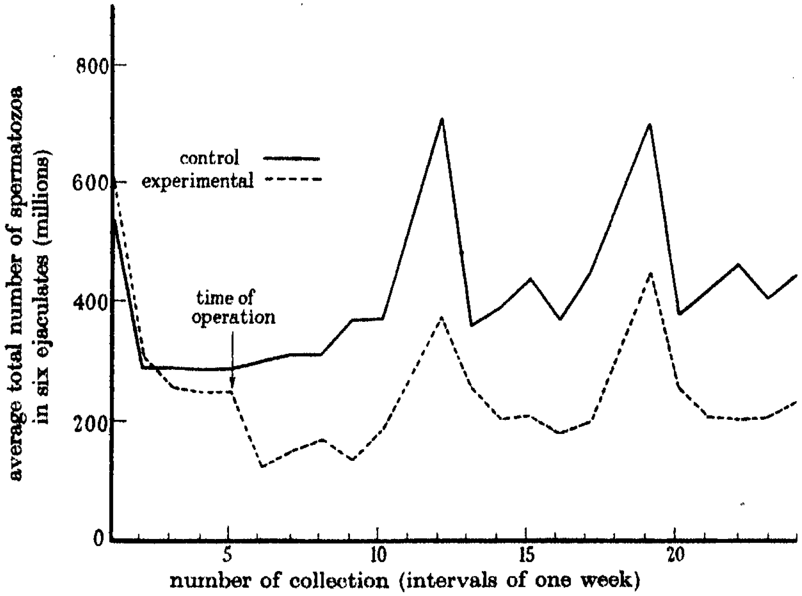

FIGURE 1. Average rate of production of spermatozoa of control and experimental bucks.

TABLE 2. VOLUMES OF SEMEN (IN C.C.) PRODUCED BY CONTROL AND EXPERIMENTAL BUCKS IN A TOTAL OF SIX EJACULATIONS COLLECTED AT INTERVALS OF 1 WEEK

No. of coll.	Buck 9 control	Buck 8 exp.	Buck 3 control	Buck 1 exp.	Buck 13 control	Buck 16 exp.	Buck 10 control	Buck 11 exp.	Average control	Average exp.	Exp. group as % of control
1	3·20	2·39	1·35	1·86	1·67	1·61	2·30	0·98	2·13	1·71	80·3
2	2·82	2·56	2·05	2·00	1·35	1·86	1·78	1·54	2·00	1·99	99·5
3	3·10	2·46	1·90	1·53	1·54	1·99	2·27	1·10	2·20	1·77	80·4
4	2·41	2·17	1·59	1·50	1·79	1·78	2·22	1·70	2·00	1·79	89·5
5	2·65	2·27*	1·25	1·89*	1·46	1·87*	2·17	1·29*	1·88	1·83*	97·3*
6	2·27	2·03	1·41	1·34	1·59	1·84	2·20	0·96	1·77	1·54	87·0
7	3·26	2·12	1·39	1·22	1·61	1·99	1·83	1·14	2·02	1·62	80·2
8	2·99	2·54	1·92	1·67	1·66	2·06	2·04	0·83	2·15	1·77	82·3
9	3·02	2·18	1·50	1·46	1·87	2·10	1·57	1·48	1·99	1·80	90·4
10	3·12	2·37	1·48	1·62	2·10	1·88	1·99	1·33	2·17	1·80	82·9
11											
12	2·86	2·59	3·07	2·22	2·20	2·43	2·99	1·56	2·78	2·20	79·1
13	3·49	2·68	1·64	1·92	2·39	1·90	2·32	1·64	2·46	2·03	82·5
14	2·73	2·49	2·21	2·01	2·33	2·16	1·71	1·98	2·24	2·26	100·9
15	2·64	3·15	1·74	1·73	2·41	2·13	2·18	1·78	2·24	2·20	98·2
16	2·08	1·93	2·38	1·71	2·50	2·04	2·10	1·46	2·26	1·78	78·8
17	3·09	2·62	2·06	2·03	2·60	1·83	1·96	1·76	2·34	2·06	84·4
18											
19	2·76	2·62	2·07	2·52	2·72	2·70	2·48	2·48	2·51	2·58	102·8
20	2·17	2·89	1·71	1·79	2·12	1·77	2·10	1·31	2·02	1·94	96·0
21	1·93	2·29	1·66	1·92	2·77	2·13	1·99	1·04	2·09	1·84	88·0
22	2·56	1·99	1·89	2·17	2·62	1·87	2·37	1·71	2·36	1·93	81·8
23	1·66	2·45	1·87	1·72	2·48	2·21	2·45	1·82	2·11	2·05	97·1
24	1·87	2·70	1·64	2·05	3·18	2·00	2·32	1·43	2·25	2·04	90·7
Total no. of ejaculations	132	132	132	132	132	132	132	132	528	528	

* One testis removed after collection made on this day. (No collections were made at the 11th and 18th weeks.)

A gradual increase in the rate of production of spermatozoa during the course of the experiment is to be noted in the performance of all bucks; as the bucks were young, although sexually mature, at the beginning of the experiment, it is thought that this increase is correlated with the growth increase over the period of 6 months. If this is accepted it is interesting to note that the increase in spermatozoa production in the unilateral castrates is of the same order as that in the controls; that is, that the factor causing the increase with age does not produce twice the effect in the former case.

THE EFFECT OF UNILATERAL CASTRATION ON THE WEIGHT OF THE TESTIS AND EPIDIDYMIS

After the tenth collection in the last day of the experiment the bucks were at once killed by coal gas. The abdomen was opened, and when the testis had been withdrawn from the scrotum the caput and cauda of the epididymis were ligatured and dissected away from it; the vas deferens was also removed, and all were weighed. These results, together with those of the testes removed from the experimental bucks at the fifth week, are given in table 3. (The vasa deferentia of the experimental bucks were not removed until the twenty-fourth week.)

TABLE 3. THE EFFECT OF UNILATERAL CASTRATION ON THE WEIGHT OF TESTIS AND EPIDIDYMIS

	Control group			Experimental group		
Item	No. of buck	Left g.	Right g.	No. of buck	Left g.	Right g.
Testis	9	3·25	3·23	8	3·60	3·25*
	3	1·77	2·00	1	2·60	2·45*
	13	3·10	2·80	16	2·45*	2·40
	10	1·90	1·90	11	2·10*	2·17
Caput	9	0·30	0·30	8	0·40	0·40*
	3	0·33	0·37	1	0·47	0·40*
	13	0·23	0·33	16	0·25*	0·33
	10	0·37	0·40	11	0·35*	0·43
Cauda	9	0·67	0·67	8	1·00	1·00*
	3	0·60	0·63	1	0·85	0·85*
	13	0·50	0·47	16	0·70*	0·63
	10	0·95	0·97	11	0·95*	0·97
Vas deferens and ampulla	9	0·35	0·37	8	0·60	0·60
	3	0·55	0·63	1	0·45	0·43
	13	0·37	0·35	16	0·30	0·30
	10	0·83	0·83	11	0·57	0·37

* Removed at fifth week of experiment; all others removed at twenty-fourth week.

In the 19 weeks following unilateral castration the remaining testis does not appear to have increased in size. In bucks 16 and 11 there is no difference in weight, and in bucks 8 and 1 the differences are small and of an order seen to exist between testes in the control bucks. The average weight of the four testes removed at the fifth week is 2·56 g. and of the four removed at the twenty-fourth week 2·69 g. The absence of compensatory hypertrophy in weight is in keeping with the results of the experiments of Lipschutz (1922), who found no hypertrophy in the size of the testis of rabbits unilaterally castrated when mature and who defined apparent hypertrophy in rabbits similarly treated when young in terms of accelerated growth of the remaining testis; its ultimate size rarely exceeded the weight of a normal mature testis. The bucks used in the present experiment were, although young, sexually mature at the time of operation.

In birds the position appears to be different. In poultry and ducks Benoit (1931) has found that complete compensatory hypertrophy occurs in birds in which one testis is removed between the age of 18 and 42 days, and Domm and Juhn (1927) have shown by operations at different ages that, although hypertrophy occurs in all cases, it is less marked as the age of the testis at the time of operation increases.

The epididymis appears also to be unaffected by unilateral castration for, as is shown in table 3, neither the caput nor the cauda of the remaining testis shows any increase in size.

THE FREQUENCY OF COLLECTION AND THE RATE OF PRODUCTION OF SPERMATOZOA

If a measure of the rate of production of spermatozoa is to be obtained it is clear that the number of collections must be sufficient to avoid loss of ageing spermatozoa by liquefaction or dissolution (Simeone and Young 1931) and to permit a steady flow of spermatozoa into and through the epididymis. In the present experiment, for example, if compensatory hypertrophy in production of spermatozoa were to result in the remaining testis the epididymis might be incapable of containing or ejaculating the increased output of spermatozoa by the testis and ejaculates, unless increased in number, might fail to reveal the change taking place.

This possibility has been tested in several ways. In the first place a comparison has been made of the numbers of spermatozoa present in the epididymis of the bucks subjected to the regular weekly collections with those found in another group of four bucks of the same age and kept under identical conditions and differing only in the fact that no ejaculates have been collected from them. In the absence of such collections it may be assumed that the numbers of spermatozoa recovered from this group indicate the numbers that can be contained in the epididymal tract, and that lesser numbers in the case of bucks on the experiment indicate adequate removal by the periodic collections.

The numbers of spermatozoa normally present in the epididymis of the bucks on the experiment were found by adding the numbers present in the ten ejaculates collected prior to killing to the numbers subsequently recovered from the epididymis. In the case of the four remaining bucks (nos. 6, 5, 14 and 4) the numbers were, of course, those actually found in the epididymis.

The results are given in table 4, and from them it will be seen that in no case do the numbers of spermatozoa found in the bucks on the experiment

TABLE 4. THE NUMBERS OF SPERMATOZOA (IN MILLIONS) PRESENT IN VARIOUS AREAS OF THE EPIDIDYMAL TRACT OF BUCKS SUBJECT AND NOT SUBJECT TO WEEKLY COLLECTIONS

No. of buck	Testis	Spermatozoa in caput	Spermatozoa in cauda	Spermatozoa in vas deferens and ampulla	Total no. in tract	Total in 10 collections	Combined total
9	Left	70	220	1	615	796	1411
	Right	64	260	0			
3	Left	36	110	7	313	354	667
	Right	48	105	7			
13	Left	38	115	3	312	557	869
	Right	37	115	4			
10	Left	15	123	0	293	258	551
	Right	17	132	6			
8	Right	67	260	15	342	393	735
1	Right	65	220	4	289	385	674
16	Left	24	53	2	79	208	287
11	Left	30	205	8	243	195	438
6	Left	66	1163	40	2516	—	—
	Right	77	1134	36			
5	Left	134	540	10*	1268	—	—
	Right	73	510	1*			
14	Left	101	505	40	1145	—	—
	Right	75	420	4			
4	Left	50	471	33	1010	—	—
	Right	47	360	49			

* Spermatozoa lost during separation of vas deferens from urethra.

exceed those present in the non-collected bucks, and that they are, in the majority of cases, considerably less. [By a criterion of rate of spermatozoa production discussed on p. 417, that of the weight of testis, buck 9 (control) and bucks 8 and 1 (experimental) should be compared with buck 6, testes weight 5·13 g.] The number of collections can therefore be considered to have been adequate.

Further evidence is provided by comparisons within the control and experimental bucks. By comparing the first weekly collections, which, since none of the bucks had previously ejaculated, represented removal of spermatozoa from a full epididymis, with the second and later weekly collections, it will be seen that the bucks possessed a storage and ejaculatory capacity greatly in excess of that required for the numbers of spermatozoa collected at weekly intervals. This was again seen to be the case on the two occasions in which an interval between collections of fourteen days was substituted for an interval of seven days; both the control bucks and the experimental bucks (now operated) were found to be capable of storing and ejaculating the almost proportionate increase in numbers of spermatozoa collected. The results, taken from table 1, are as follows (table 5):

TABLE 5. THE EFFECT OF VARYING INTERVAL BETWEEN COLLECTIONS ON NUMBERS OF SPERMATOZOA

Period	Average production of control group millions spermatozoa	Average production of experimental group millions spermatozoa
1st collection	543	605
2nd collection	287	319
10th collection (weekly interval)	367	182
12th collection (fortnightly interval)	709	375
17th collection (weekly interval)	453	197
19th collection (fortnightly interval)	702	453

It seems very likely, therefore, that if unilateral castration had resulted in an increased output of spermatozoa by the remaining testis a single epididymis would have been capable of containing and ejaculating it.

Finally, there is the evidence provided by the correlation found to exist between the weight of the testis and the rate of production of spermatozoa (p. 417). It is unlikely that the nature of this relationship could have been defined if, for any reason, the number of collections had not been sufficient to account for all or most of the spermatozoa being produced.

WEIGHT OF TESTIS AND NUMBERS OF SPERMATOZOA PRODUCED

A significant positive relationship is found to exist between the weight of the testis and the number of spermatozoa which it produces. The regression of numbers of spermatozoa produced on weight of testis has been calculated from the results in table 6; the weights of the two testes have been added in the case of the control bucks and the figure for the number of spermatozoa produced is obtained by averaging the weekly total number of spermatozoa in eight ejaculations (omitting collections 8 and 10 because of their very small volumes) in the 4 weeks prior to killing. The regression formula is:

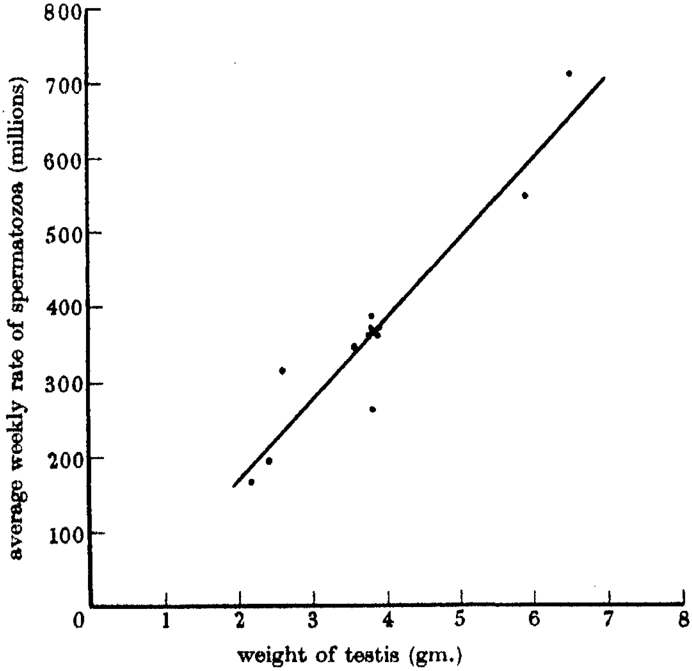

FIGURE 2. Relation between weight of testis and rate of spermatozoa production.

$$E = 365 + 109 \cdot 21\ (x - 3 \cdot 48),$$

$$Sxy \pm 1 \cdot 911,$$

and the regression line together with the individual values in the distribution are given in figure 2. The regression is highly significant. In the case of the bucks used in this experiment it appears that 1 g. of testis is producing roughly 100 million spermatozoa per week.

TABLE 6. RELATION BETWEEN WEIGHT OF TESTIS (EXPERIMENTAL BUCKS) OR TESTES (CONTROL BUCKS) AND AVERAGE WEEKLY RATE OF PRODUCTION OF SPERMATOZOA FOR 4 WEEKS PRIOR TO KILLING

No. of buck	Wt. of testis or testes (g.)	Av. no. spermatozoa produced (millions)
11 (*E*)	2·17	165
16 (*E*)	2·40	195
1 (*E*)	2·60	314
8 (*E*)	3·60	345
3 (*C*)	3·77	385
10 (*C*)	3·80	261
13 (*C*)	5·90	546
9 (*C*)	6·48	709
	Average 3·84	Average 365

E = experimental; *C* = control.

THE RATE OF DESCENT OF SPERMATOZOA IN THE EPIDIDYMIS

From the facts in table 4 an estimate of the time taken by the spermatozoa to traverse the epididymal tract can be made by comparing the numbers of spermatozoa present in weekly collections with the numbers that remain. It is not to be expected that the numbers recovered from the tract necessarily indicate the spermatogenic activity of the testis because, as is shown in table 3, there is no evidence of a correlation between the weight of the testis and the weight of the cauda which is the part of the tract possessing the largest storage capacity. The numbers found are likely to depend therefore both on the level of spermatogenic activity and the capacity of the tract.

With this reservation, it will be seen (table 4) that in the cases of bucks 3, 10, 8 and 11 the numbers of spermatozoa withdrawn in ten collections prior to killing are roughly the same as those remaining in the tract. These latter, since the weekly numbers of spermatozoa collected were fairly constant (table 1), would presumably have been recovered at the next weekly collection and a like number would have moved into the epididymis. The rate of passage through the epididymis would therefore have been accomplished in about 7 days. In the case of the remaining bucks, and especially bucks 13 and 16, the time taken must be much less for the numbers of spermatozoa remaining after ten collections are much smaller than those removed. A more rapid rate of descent, accomplished perhaps in 3–4 days, must therefore be possible.

These estimates are considerably shorter than those made by Toothill and Young (1931) for the rate of descent of spermatozoa in the epididymis of the guinea-pig. The technique they employed consisted of injecting sterile India ink particles into the caput of the epididymis and observation of the time taken for the particles to appear in the ejaculate. In sexually inactive males particles were recovered in the lower part of the epididymis in about 14–18 days; in males made to ejaculate by means of an electrical stimulus, in about 12–16 days, and in males in which the connexion between the testis and caput was ligatured at the time of injection, in 21–37 days. Gunn (1936) employed this technique with rams and found it unsatisfactory because of the difficulty of injecting the ink particles into the lumena of the tubules. Ink particles unavoidably injected into the walls of the tubules appeared as lumps in the ejaculate when the walls broke down. This difficulty was overcome by injecting ink into the rete of the testis. In animals so treated and made to ejaculate by an electrical stimulus ink particles appeared in 5–6 days. (From these observations the frequency of ejaculation employed is not clear.)

Phillips and MacKenzie (1934) have based an estimate of rate of descent on the time required for the appearance of abnormal forms of spermatozoa in the ejaculate of rams in which the spermatogenic activity of the testis has been impaired or is in process of being destroyed by heat treatment. In such cases abnormal forms appeared in 4–13 days in rams allowed three services daily on every third day. Gunn questions the validity of such a method of estimation because he has found that heat treatment may also produce abnormal forms of spermatozoa among those already present in the epididymis at the beginning of the treatment.

Because of the apparent deficiencies of these methods of estimation it is suggested that the method described in the present experiment—that of relating the numbers of spermatozoa present in the ejaculates to the numbers remaining in the tract of males subjected to regular collections—might be employed in experiments designed to discover the rate of descent especially with respect to its relation to frequency of ejaculation.

DISCUSSION

The most significant finding is the fact that unilateral castration does not cause compensatory hypertrophy of function in the remaining testis. This is in sharp contrast to the behaviour of the ovary. Asdell (1924) has shown that if one or one and one-half ovaries are removed in a mature female rabbit complete compensatory hypertrophy of function takes place rapidly in the remaining ovarian tissue; the numbers of ova shed are equal to those previously produced by two ovaries. It is now generally agreed that these numbers are determined by the level of gonadotropic hormones in the blood. Smith and Engle (1927) have demonstrated the close relationship that exists between the anterior pituitary gland and the functioning of the testis in the rat; removal of the gland stops spermatogenesis. Smith (1930) has also shown that implantation of pituitary gland in hypophysectomized rats will enable the testis to resume normal function. While this relationship is thus clearly established the results of the present experiment suggest that the effect of the pituitary gland is not quantitative. The evidence of the effect of unilateral castration, of the relationship between the weight of the testis and its rate of spermatozoa production (p. 417), together with that of the growth effect (p. 413) suggest that the testis itself exercises ultimate control on the number of spermatozoa produced. Further experiments are being designed to test this hypothesis and to attempt to find the nature of the control.

I am most grateful to Dr J. Hammond, F.R.S., for his interest and criticism in the course of this work.

SUMMARY

The effect of unilateral castration in the sexually mature rabbit on the production of spermatozoa has been studied quantitatively by means of semen exhaustion tests.

The frequency of collection is shown to have kept pace with the rate of production of spermatozoa by the testis.

Contrary to what occurs in the ovary there is no hypertrophy of spermatogenic function or of weight of testis following unilateral castration.

There is a highly significant positive relationship between the weight of the testicular substance and the numbers of spermatozoa which it produces.

The passage of spermatozoa through the epididymis may be accomplished in 4–7 days.

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The breakdown and synthesis of starch by an enzyme system from pea seeds

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INTRODUCTION

The gradual elucidation of the intermediary role of phosphorylated sugars in the breakdown of carbohydrate by the enzymes of yeast and certain animal tissues forms an outstanding achievement in the field of the biochemistry of carbohydrates. It is attractive to suppose that the rapid interconversions of carbohydrates which occur in the higher plants may depend also upon the formation of labile phosphorylated derivatives of sugars. It cannot be claimed, however, that this view is supported by any extensive body of facts. Since so far as the higher plants are concerned, only fragmentary observations have been recorded on the occurrence and nature of phosphorylated sugars; relatively little is known, moreover, concerning the types of enzymic mechanism which may be present to act upon such esters. Investigations to be described in this and subsequent communications are directed towards various aspects of this metabolic problem.

In the present paper it is proposed to consider a system of enzymes extracted from mature pea seeds which illustrates one type of mechanism by which phosphoric esters are formed in vegetable tissues. Further, the study of this system has disclosed the mechanism by which starch is synthesized in the plant.

The earliest demonstration by Bodnár (1925) of the existence of a phosphorylating enzyme in a higher plant was based on experiments with this same tissue. On suspending flour from ground peas in a phosphate buffer, Bodnár found that a progressive disappearance of inorganic phosphate occurred during incubation. More recently, Tankó (1936) investigated the fate of the phosphate which disappears in this system and was able to recover the main portion of it (about 90 %) in the form of fructofuranose-1 : 6-diphosphate. Hexose-monophosphates were also present, of which a fraction was isolated which resembled the mixture of 6-phosphoric esters of glucose, fructose and mannose isolated by Robison (1922, 1932) from the products formed by the phosphorylation system of yeast. From these investigations it became clear that pea seeds contain an enzyme system which catalyses the phosphorylation of some carbohydrate constituent originally present in the tissue.

For a closer analysis of the reactions involved in this system it appeared essential that the enzyme should be separated from the tissue and that the carbohydrate substrate for the esterification process should be identified. Experiments along these lines have shown that, in the presence of inorganic phosphate, starch and a variety of starch dextrins are readily phosphorylated under the action of extracts from pea seeds but that, with the exception of maltose which is acted upon only very slowly, simple sugars are not attacked.

The first recognizable step in the phosphorylation of starch is the formation of the non-reducing acid-labile glucose-1-phosphate. This ester has been obtained in high yield in the form of the crystalline potassium salt. It appears to be identical with the glucose-1-phosphate which was first isolated by Cori and Cori (1936) as the primary product of the esterification of glycogen in muscle extracts, and later described by Kiessling (1938).

Of considerable botanical interest is the fact that the conversion of starch to glucose-1-phosphate is a reversible reaction: this is catalysed in both directions by an enzyme which will be referred to as a phosphorylase. When glucose-1-phosphate is added to an extract of peas a portion of it is converted into starch with the liberation of free phosphate. The reversibility of the initial stage of the phosphorolytic breakdown of glycogen has recently been reported by a number of authors working with preparations of phosphorylase from yeast—Schäffner and Specht (1938), Kiessling (1939); muscle—Cori, Schmidt and Cori (1939); and liver—Ostern and Holmes (1939).

Glucose-1-phosphate, however, undergoes an alternative transformation which under certain conditions leads to its rapid and practically complete disappearance from the system. In this reaction reducing hexose-monophosphates, resistant to acid hydrolysis, are formed; this transformation takes place in at least two distinct stages catalysed by different enzymes, but for the present these will be referred to merely as the phosphoglucose conversion system.* The products of the action of this system upon glucose-1-phosphate consist, to a considerable extent at least, of a mixture of glucose-6-phosphate, fructose-6-phosphate, and mannose-6-phosphate.

As mentioned earlier, Tankó observed the accumulation of hexose-diphosphate in suspensions of pea flour to which phosphate was added. It is found that the addition of dissolved starch to such a suspension greatly increases the rate of the esterification of phosphate, and that the main product under suitable reaction conditions is hexose-diphosphate as in Tankó's experiments. This ester is also formed when crude aqueous extracts are used, but in extracts which have been subjected to prolonged dialysis little or no hexose-diphosphate is formed. The formation of glucose-1-phosphate from starch and inorganic phosphate, and the subsequent conversion of this ester to the reducing 6-esters, occurs readily in such dialysed extracts, but it seems likely that the formation of hexose-diphosphate (presumably by the conversion of fructofuranose-6-phosphate into fructofuranose-1 : 6-diphosphate) requires the presence of a dialysable co-enzyme.

The experiments which will be presented below constitute a general survey of this complex set of reactions. It is proposed to describe later more detailed studies on the separate components of this system.

METHODS

Preparation of extracts

Pea seeds of the variety Laxton's Progress (a wrinkled culinary variety) were ground to a fine flour in a C. and N. mill. The freshly ground flour was then extracted several times with ether on a Buchner funnel to remove the bulk of the fat and, after removal of the ether, the flour was suspended in either three or four times its weight of distilled water, powdered thymol or a layer of toluene being added. Extraction was continued for 24 or 40 hr. at 2° C, the suspension being stirred occasionally; the longer period was found to yield a slightly more active extract. After centrifuging off the solid matter the supernatant fluid was filtered.

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* The presence in pea extracts of one of the component enzymes was demonstrated by Tankó (1936); this enzyme, referred to as a phosphohexokinase, catalyses the reversible interconversion of glucose-6-phosphate, fructose-6-phosphate and mannose-6-phosphate.

Trials with different extractants (e.g. 1 % potassium chloride solution, 6 % ammonium sulphate, 1 % disodium phosphate, sodium bicarbonate at pH 7·5) failed to show any advantage over the simple extraction with water at the natural acidity of the tissue (about pH 6·2) which was adopted for routine purposes.

The crude aqueous extract contains normally about 18–20 % of the original dry matter of the peas. It is opaque and creamy in appearance, and when stored a fine-grained sediment of protein forms in a day or two, and this deposition continues for several weeks.

Dialysis against distilled water (saturated with thymol) was carried out at 2° C in narrow thin-walled sacs of cellophane. The total period of dialysis was usually 24 hr.; during the first 8 hr. sacs containing 500–700 ml. extract were dialysed against about 30 l. water (in six changes) and then against running distilled water for the remaining period, the water being stirred continuously by the bubbling of a stream of air.

A precipitate which formed during dialysis was removed by centrifuging. The dialysed extract, although it contained normally only about one-third of the dry matter present in crude extract, was still opaque, and on storage a deposition of protein occurred as in the case of crude extracts.

It was desirable to eliminate this progressive deposition of protein for a number of reasons, chief of which was the fact that a considerable proportion of the phosphorylase was adsorbed by the precipitate. Thus on centrifuging an extract which had been held for 14 days at 2° C, the supernatant fluid had only 5 % of the original activity; when, however, the entire suspension was added to a digest (at pH 7·5) there was a marked induction phase, due presumably to the gradual elution of the enzyme from the suspended protein, after which the reaction proceeded with approximately 90 % of the original velocity.

It was found on treating dialysed extracts with kaolin (15–25 g. kaolin per 100 ml. extract, with stirring for 10 min., followed by gravity filtration) that clear yellowish filtrates were obtained which retained almost the whole of the phosphorylase activity and in which no sedimentation occurred during storage. About 50 % of the dry matter present in the dialysed extract was removed by the kaolin, and such cleared extracts contained normally 16–20 mg. dry matter per ml.

The phosphorylase activity was found to decrease relatively slowly during storage at 2°, losses of the order of 30–50 % occurring in 10 days.

The phosphoglucose conversion system was considerably more labile, and it was frequently found to be completely inactive after 10–14 days' storage.

Determination of phosphorus

Phosphorus in the form of orthophosphate was determined by the method of King (1932), the concentrations and volumes of the reagents being suitably altered for use with a final volume of 25 ml. The colorimetric readings were made by means of an absolute photometer, extinction coefficients for light passing through a deep red filter being determined in cells of appropriate depth. The intensity of the blue colour (due to the reduction of phosphomolybdic acid) reaches a value within 20 min. which remains without detectable change for a further 15 min.; accordingly, as routine, the readings were made during this interval.

Total phosphorus was determined after combustion with perchloric acid; phosphorus in the form of acid-labile phosphoric esters was determined after heating for 7 minutes at 100° in the presence of 1 N perchloric acid; by this treatment glucose-1-phosphate, for example, is completely hydrolysed. For convenience, the following abbreviations will be used to designate the different categories of phosphorus compounds: *free-P*, phosphorus in the form of free orthophosphate; *organic-P* which corresponds to the increase in *free-P* on combustion; 7-*min.-P* which corresponds to phosphate liberated during the hot acid treatment described above.

The progress of esterification of phosphate in enzymic digests was followed by different methods. The simplest (method 1) was to determine the decrease in *free-P* in the reaction mixture. Samples of appropriate volume were delivered at intervals into trichloracetic acid solution of such volume and strength as to give a final concentration of 7–8%; the precipitated protein was filtered off after a few minutes, and determinations of *free-P* were made on suitable volumes of filtrates. The usefulness of this method is restricted to digests with relatively low initial concentrations of phosphate, since with high concentrations, even though the amount of phosphorus esterified is greater, the percentage decrease in *free-P* becomes too small to be followed precisely.

In order to follow the increase in *organic-P* in digests with relatively high concentration of inorganic phosphate it was necessary to introduce some treatment for removing the excess of inorganic phosphate. No simple method suitable for routine observations has been encountered by which it is possible to obtain a quantitative separation of all classes of hexosephosphates from relatively large amounts of inorganic phosphate. In the case of hexose-monophosphates (including glucose-1-phosphate) it was found that by treatment with magnesia mixture under carefully controlled conditions inorganic phosphate could be completely precipitated with only very small losses of these esters; under the same conditions, however, fructose-diphosphate was found to be largely carried down by the precipitate. The inherent difficulty in the separation of fructose-diphosphate from an excess of inorganic phosphate has not proved, however, to be a serious disadvantage in the present study, and considerable use has been made of two procedures based on the removal of inorganic phosphate by magnesia mixture, these being used for following esterification under conditions in which hexose-monophosphates alone are formed.

Thus in one procedure (method 2) a portion of the trichloracetic filtrate (obtained as described above) was neutralized and subjected to treatment with magnesia mixture under the conditions devised by Kursanov (1938). After removal of the precipitate, the *organic-P* was determined on a suitable volume of the filtrate.

Method 3 is a convenient simplification of method 2, the digest sample being run directly into an appropriate volume of a diluted magnesia mixture.* It was found that on adding 1–3 ml. of solutions similar in composition to typical samples from digests (containing 0·5–1·5 ml. dialysed enzyme preparation, inorganic phosphate varying from 0·02–0·10 M and known amounts of the different hexose-monophosphates) that recoveries of hexose-monophosphate-P were approximately 95 %. In contrast when fructose-diphosphate was added, only small amounts (10–30 %) were recovered in the filtrate. It may be mentioned that about 90 % of the small amount of phosphorus present in purified enzyme preparations was precipitated by magnesia in the presence of the amounts of inorganic phosphate mentioned.

By means of methods 2 and 3 it has been possible to survey the reactions catalysed by dialysed extracts in the presence of concentrations of phosphate up to 0·2 M, and as will be seen the main product which accumulates under these conditions is glucose-1-phosphate. Although the data obtained by these methods are remarkably consistent the results cannot be claimed to be strictly quantitative, since small losses of the order of 3–7 % are known to occur, for which no corrections have been made. The general validity of all essential points has been established by independent means, e.g. by the isolation of the products.

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* For example, 1 or 2 ml. samples of digest, containing amounts of inorganic phosphate up to 0·2 M, were delivered into 10 ml. of a solution having the following composition: 0·165 % magnesium chloride (hexa-hydrate), 0·21 % ammonium chloride, 2·5 % ammonia.

In the case of digests with crude non-dialysed extracts the difficulties of evolving a satisfactory system of rapid micro-analysis are so great that this has not been attempted. In such digests the progress of esterification was followed by method 1 and the character of the products determined after their isolation in the form of the barium salts.

Conditions of reactions

Experiments were carried out at 25° C. The required volume of enzyme (normally $\frac{1}{4}$ to $\frac{1}{2}$ of the final volume of the digest), and the remaining components of the reaction mixture were brought to this temperature in separate vessels. The contents were then mixed and the vessels thoroughly rinsed with the mixture. The first sample was then withdrawn and delivered into the appropriate reagent, the delivery being started usually 0·7 min. after the initiation of the reaction. Subsequent samples were taken to define the progress curve, and from this the composition of the digest at zero time was calculated by applying a slight correction to the value obtained for the 0·7 min. point.

Usually in digests with high phosphate concentration no additional buffer was added; the phosphate buffers used were prepared from K_2HPO_4 and NaOH. When the phosphate concentration was low, the acidity of the enzyme was previously adjusted to the desired pH, and in some experiments veronal-acetate buffers were added.

Soluble starch was used as substrate in many experiments; this was prepared by the method described earlier (Hanes and Cattle 1938).

All solutions to be used as constituents of enzymic digests were kept saturated with toluene or thymol, and a layer of toluene was added to the digests themselves.

EXPERIMENTS

(*a*) *Carbohydrate substrates for the phosphorylase of peas*

When dialysed extracts of peas are incubated at 25° either with or without the addition of inorganic phosphate, extremely small and slow changes may be observed in the partition of phosphorus compounds as determined by the different analytical methods (1–3). It will be evident from data given in ensuing experiments that these changes are negligibly small compared with those observed when a suitable carbohydrate substrate together with inorganic phosphate is added. Non-dialysed extracts, when freshly prepared, exhibit a slight esterification, which is presumably due to the presence of a trace of carbohydrate substrate. This disappears either on storage or on dialysis.

In the experiments to be considered in this section relatively high concentrations of inorganic phosphate were added, the initial concentrations in the reaction mixtures amounting to 0·1 M or more. Under these conditions, as will be shown later, the activity of the phosphorylase, leading to the formation of glucose-1-phosphate, is high, whereas the subsequent conversion to hexose-6-phosphates is strongly inhibited.

Observations are given in table 1 on a series of digests to which soluble starch and different sugars were added. The enzyme was a dialysed extract, 10 ml. being added to each digest of 20 ml. The initial phosphate concentration was 0·125 M and the pH 7·5.

TABLE 1. THE EFFECT OF ADDING SOLUBLE STARCH AND VARIOUS SUGARS TO DIALYSED EXTRACT OF PEAS IN THE PRESENCE OF PHOSPHATE

Digests of 20 ml. total volume; initial phosphate, 0·125 M; pH, 7·5; enzyme, 10 ml. dialysed extract. Determinations of *organic-P* by method 2.

Digest no.	Carbohydrate added	Increase in *organic-P* (mg. P per 20 ml.) 30 min.	180 min.	1380 min.
1	None	−0·04	0·06	0·12
2	Starch 90 mg.	0·61	3·60	7·43
3	Glucose 400 mg.	−0·04	0·02	0·10
4	Fructose 400 mg.	0·01	0·02	0·19
5	Sucrose 400 mg.	−0·05	−0·03	0·16
6	Maltose 365 mg.	0·30	1·16	2·46
7	Starch 90 mg.	0·62	3·58	7·45
8	Starch 90 mg. + glucose 400 mg.	0·53	3·06	5·24
9	Starch 90 mg. + fructose 400 mg.	0·50	3·30	7·83
10	Starch 90 mg. + sucrose 400 mg.	0·60	3·21	6·86
11	Starch 90 mg. + maltose 365 mg.	0·54	2·92	8·84

It is clear that when soluble starch was added (no. 2) considerable esterification of phosphate occurred, whereas with additions of glucose, fructose or sucrose (nos. 3–5) the changes in *organic-P* were small and of the same order as those occurring without added carbohydrate (no. 1). With maltose (no. 6), however, appreciable esterification took place, although the velocity and amount of the esterification were much smaller than with starch, despite the approximately fourfold difference in the concentration of these substrates.

In a continuation of this experiment, the effect of the presence of these different sugars together with soluble starch was examined. Glucose and fructose in 2% concentration (nos. 8 and 9) both caused an appreciable inhibition initially, but whereas the glucose effect became more pronounced in the course of the reaction, the inhibition by fructose became smaller and there appeared finally to be a slightly increased esterification (cf. also no. 4). With added sucrose (no. 10), a very slight initial inhibition increased progressively. (It is possible that the sucrose was being hydrolysed during the period, but this was not determined.) In the case of maltose (no. 11) the velocity in the early stages was intermediate between the values for starch and maltose separately, but the final value exceeded considerably that observed for starch alone; it was rather less than the sum of the values for the two substrates added separately—8·8 mg. as compared with 9·9 mg. phosphorus.

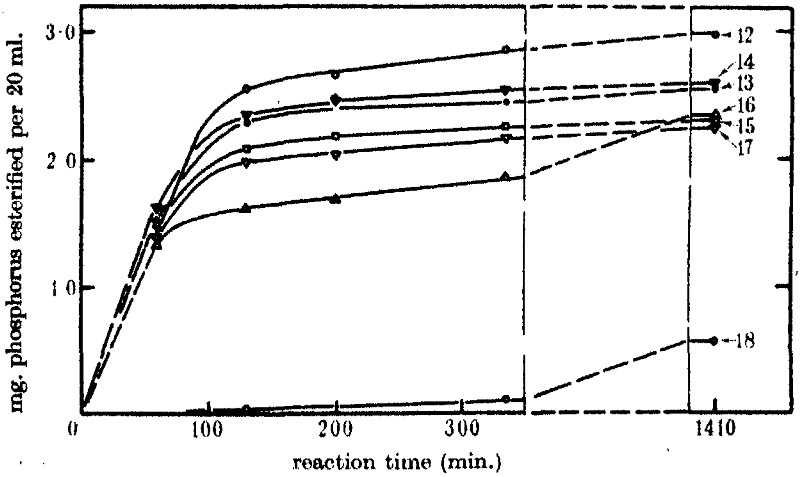

FIGURE 1. The progress of esterification with additions of different starch products (30 mg. per 20 ml. digest); 0·1 M phosphate initially, pH, 6·8. Determinations by method 3. Digest no. 12, starch paste; 13, soluble starch; 14 and 15 (respectively), red-violet staining and achroic dextrins (formed by α-malt-amylase); 16, rose-staining dextrins (formed by amylase of peas); 17, α-amylodextrin (formed by β-malt-amylase); 18, maltose.

In the next experiment (figure 1) a comparison was made of the esterification which occurred when a range of starch products was added as substrates. These included starch paste, soluble starch, a variety of dextrins, and maltose, each of which was added in the same concentration (30 mg. per 20 ml. digest). The enzyme was a dialysed kaolin-treated extract of which 10 ml. was added per 20 ml. digest.

The starch paste was prepared from ether-washed potato starch.

Two of the dextrin substrates were prepared by subjecting starch paste to the action of a highly purified preparation of α-malt-amylase. In one case the action was arrested when the iodine colour had become red-violet; in the other it continued to the stage when there remained no iodine-staining dextrins. The reducing powers of these two dextrin mixtures, respectively, were found to be 7·6 and 16·4 (glucose = 100), corresponding to mean values for chain lengths of 13 and 6 glucose units. On the basis of previous experience in the fractionation of the products of action of α-malt-amylase, it may be stated that no undegraded starch remained in either the red-violet staining or the achroic dextrin preparations, and that maltose (and glucose) were virtually absent from both. In both cases they consisted of disaggregated fragments of the chain molecule of starch terminated by free reducing groups, i.e. reducing dextrins.

Another dextrin mixture was prepared by utilizing the amylase of moderate activity which is present in the pea phosphorylase preparation itself. This was done by adding the appropriate amount of soluble starch to the extract in the absence of phosphate. After 95 min. at 25°, phosphate was added, and observations on the phosphorylation system begun. By this time, as was determined on a control digest, the iodine colour had changed to red, and the reducing power of the dextrins was 14.

Finally, α-amylodextrin, which exemplifies a class of non-reducing dextrin in which the molecular chains retain a highly aggregated condition, was added. α-Amylodextrin represents a fragment of the chain molecule of starch (with a chain length of 11–12 glucose units) which remains after the completed action of β-malt-amylase on starch and which after isolation is resistant to hydrolysis by this amylase (Haworth, Hirst, Kitchen and Peat, 1937; Hanes 1937).

Progress curves showing the esterification of phosphorus in these digests are plotted in figure 1. It is clear that the phosphorylase from peas is able to act upon all of these varied products from starch, although in the case of maltose (no. 18) the amount of phosphorus esterified was small, the change during the first 335 min. being barely significant.

With starch paste (no. 12) the initial rate of the reaction was slightly lower than that occurring with soluble starch (no. 13) and certain of the dextrin mixtures, but the final extent of conversion exceeded that observed with all other substrates. The red-violet staining dextrin (formed by α-malt-amylase) and soluble starch (nos. 14 and 13) exhibit almost identical progress curves; and again, at a lower level of conversion, the achroic dextrin mixture (formed by α-malt-amylase) (no. 15) and α-amylodextrin (no. 17) gave somewhat similar results. The rose-staining dextrin mixture formed by the action of the pea amylase (no. 16) was esterified more slowly than the other dextrins, but the final conversion was about the same as that for the achroic dextrin mixture (no. 15).

In addition to the range of substrates for which data have been given above it has been found that esterification also occurs with additions of glycogen. Experiments with different specimens of glycogen, however, have given such variable effects as regards the rate and extent of conversion that the presentation of the data will be deferred until more observations are available.

These results indicate that the phosphorylase from peas is able to act upon a much wider range of carbohydrate substrates than is generally believed to be the case with the phosphorylases from muscle and yeast (cf. Parnas 1937; Euler and Bauer 1938). These latter enzymes in fact are said to exhibit a fairly strict specificity for undegraded glycogen and starch.

In the case of the phosphorylase from peas it seems that almost any saccharide composed of α-glucopyranose units linked in positions 1 and 4 (as in maltose) may serve as substrate, regardless of the length of the chain and whether or not the saccharide chains exist in the form of highly colloidal aggregates or in the free form terminated by reducing groups. A consideration of the results so far available suggests that the rate and extent of the conversion of oligo-saccharides is appreciably less than that of longer and more complex chain fragments.

(*b*) *The breakdown of starch in the presence and absence of phosphate*

Using a rigorously dialysed extract of peas which contained virtually no inorganic phosphate (less than 0·01 mg. *free-P* per ml.), a comparison was made of its action on soluble starch at pH 7·5 without added phosphate and in the presence of 0·1 M phosphate (digests 19 and 20). It may be assumed that in the former the breakdown of starch which occurred was exclusively hydrolytic in character, catalysed by the amylase system present in the extract; in the latter, the breakdown was due to the combined action of the amylase and the phosphorylase.

In this experiment the action in digest samples was arrested, and proteins were precipitated by delivery into boiling water; after cooling, a small amount of acetic acid was added, the samples were brought to 25 ml. and then filtered.

Observations made on aliquots of such filtrates from the two digests are given in table 2 and in figure 2. These include determinations of the increase in reducing power (by a copper reagent of the type described by Somogyi (1937)) and of the extent of esterification of phosphate (by method 2). Finally, quantitative measurements were made on the iodine colouring property by the procedure described elsewhere (Hanes and Cattle 1938). In the present experiment the measurements of light absorption were made on aliquots containing the equivalent of 5 mg. of starch (or the products

TABLE 2. THE BREAKDOWN OF STARCH IN THE PRESENCE AND ABSENCE OF PHOSPHATE

Digests of 50 ml., containing 150 ml. soluble starch; pH, 7·5; enzyme, 20 ml. extract, dialysed 36 hr.

	Digest no. 19 (without phosphate)		Digest no. 20 (with 0·1 M phosphate)			
Reaction time min.	Reducing power* %apparent glucose	Iodine colour† starch = 100	Reducing power* %apparent glucose	Iodine colour† starch = 100	Increase in *organic-P* mg./20 ml.	Ratio *7-min.-P/total organic-P*
15	2·9	87	2·0	77	0·50	—
35	5·4	83	4·2	58	0·92	1·02
90	12·1	60	11·3	26	2·07	0·99
195	22·5	28	21·0	2	3·94	1·00
360	32·5	8	29·4	—	4·94	0·98
1500	61·7	—	74·7	—	5·00	0·62
3180	70·4	—	87·5	—	5·18	0·26

* The increase in reducing power by reagent of Somogyi (1937) type, expressed as percentage apparent conversion of the substrate into glucose.

† The observed value of E_{57} expressed as a percentage of the initial value (cf. figure 2).

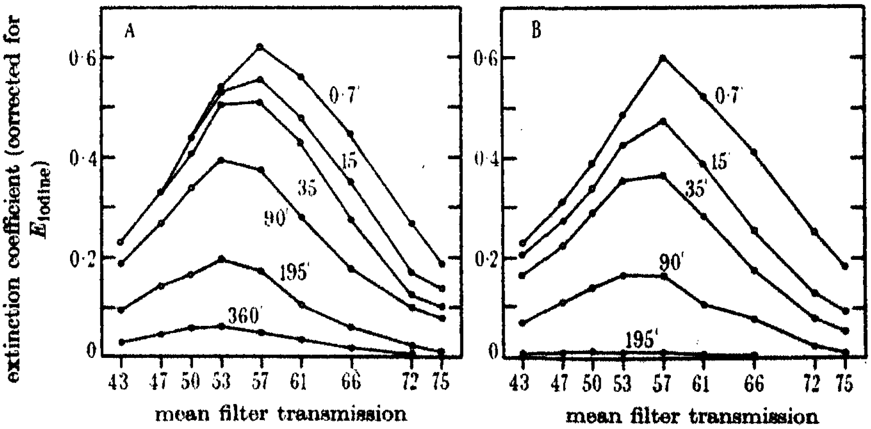

FIGURE 2. Alterations in iodine coloration: A, in digest 19 (without phosphate); B, in digest 20 (with 0·1 M phosphate). Reaction times are indicated for the different extinction-λ curves.

from this amount) diluted to 100 ml. after the addition of 10 mg. iodine with 15 mg. of potassium iodide. As in previous work, the absorption values are expressed in terms of the *increase* in the extinction coefficients which results from the formation of the coloured polysaccharide-iodine complex.

To consider first digest 19 (without phosphate), certain deductions may be drawn regarding the nature of the amylase system present in the extract. The transformations in the extinction-λ curves (figure 2A) are of the type which occurs during the action on starch of amylases of the α- or dextrinogenic class. Thus a closely similar sequence of changes is observed during the action of α-malt-, *Aspergillus*, salivary, and pancreatic amylases (cf. Hanes and Cattle 1938), there being initially a considerable fall in the E-values toward the red end of the spectrum while the absorption in the violet and blue remains unchanged; later the values decrease over the whole spectrum but more rapidly in the longer wave-lengths.

It is clear from the increase in reducing power that the substrate is converted ultimately into extremely short chain fragments including an appreciable proportion of glucose. Thus the observed reduction values after 360, 1500, and 3180 min. correspond respectively to the liberation of 1 reducing group per 3, 1·6 and 1·5 glucose units of the original substrate. These facts, together with preliminary observations on the dextrins which are formed, suggest that under the action of the pea amylase starch is rapidly disaggregated and is then cleaved into chain fragments terminated by free reducing groups. These reducing dextrins, complex at first and exhibiting blue or violet colours with iodine, are further fragmented yielding red- or orange-staining dextrins and finally achroic products. The hydrolysis continues long after the achroic stage is reached.

In the corresponding digest with added phosphate (no. 20) extensive esterification occurred (table 2). The increase in *organic-P* proceeded steadily until it amounted to approximately 5 mg. P per 20 ml. digest, after 360 min. reaction. At this time practically the whole of the esterified-P was in the acid-labile form, but on prolonged incubation, e.g. 1500 and 3180 min., the ratio *7-min.-P/organic-P* fell from 0·98 to 0·62 and 0·26, respectively. Anticipating to some extent the proof afforded in subsequent sections, it may be stated that glucose-1-phosphate formed the exclusive product of the esterification up to 6 hr., but that at the later stages appreciable proportions of this ester had been converted into the reducing hexose-6-phosphates.

The esterification of 5 mg. P with the formation of hexose-monophosphate requires 29 mg. hexose (or 26·1 mg. anhydrohexose). Since 20 ml.

of digest contained initially 60 mg. starch, the extent of its transformation into glucose-1-phosphate amounted to 43·5 %.

Accompanying this conversion of a considerable portion of the substrate into the non-reducing glucose-1-phosphate (under the action of the phosphorylase), it is clear from the reduction values that the action of the amylase was but little affected by the addition of 0·1 M phosphate. Thus in the first 6 hr. the increases in reducing power in digest 20 were only slightly lower than those observed in digest 19. Later, however, when appreciable amounts of glucose-1-phosphate had been converted into the reducing hexose-6-monophosphates, the reduction values in digest 20 considerably exceeded those in digest 19. It will be noted that almost the whole of the substrate was converted ultimately into hexose units—partly in the form of hexose-monophosphate * and partly free glucose.

Despite the fact that the rate of action of the amylase was approximately the same in the two cases, it is clear that the rate of alteration in the iodine-colouring property was greatly accelerated by the addition of phosphate. It follows, therefore, that the breakdown process catalysed by the phosphorylase causes *per se* a transformation of the iodine colour, indicating a direct attack upon complex iodine-staining dextrins.

The type of alteration in the iodine-colouring property resulting from the action of the phosphorylase is a matter of interest. This is shown clearly by a comparison of the extinction-λ curves for the two digests in figure 2. In the presence of phosphate, not only did the extinction values fall more rapidly, but the type of change in the extinction-λ curves is different. Thus from the early stages of the reaction, the E-values decrease over the whole spectrum.

This type of alteration in the extinction curves had previously been encountered only during one particular type of enzymic decomposition of starch, namely, that occurring under the action of the saccharogenic (β) malt amylase, and it is possible to reproduce extremely closely the sequence of changes observed in digest 20 by degrading starch under the action of an appropriate mixture of β-malt-amylase together with an amylase of the α-dextrinogenic type.

Evidence has been advanced elsewhere which shows that the degradation of starch under the action of β-malt-amylase consists exclusively of an end-wise attack in which maltose is liberated by the cleavage of successive terminal pairs of glucose units from the non-aldehydic end of the molecular chain; and further that the peculiarities in the resultant changes in the iodine-colouring property are a reflexion of this purely terminal type of degradation (Hanes and Cattle 1938). The present observations thus suggest strongly that the action of the phosphorylase upon starch and dextrins consists also of an end-wise attack at one end of the chain structure with the liberation of the individual glucose units in the form of glucose-1-phosphate.

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* Since the reducing power of the hexose-6-phosphates is probably less than that of the equivalent amount of free hexoses, as judged by the values obtained by Robison (1932) who used an alkaline ferricyanide reagent, the actual conversion level may have been slightly higher than indicated by the final reduction value.

This conclusion is in harmony with the hypothesis advanced by Parnas (1937; cf. also Cori, Colowick and Cori 1938) in explanation of the formation of glucose-1-phosphate from glycogen under the action of the phosphorylase of muscle; according to this hypothesis the transformation consists essentially in a process of "phosphorolysis" in which the ions H^+ and $(H_2PO_4)^-$ or $(K_2PO_4)^-$ take part directly in the cleavage of glucosidic linkages (in a manner analogous to the hydrolysis of such linkages by reaction with H^+ and OH^-).

Thus, assuming the constitution of glucose-1-phosphate to be α-glucopyranose substituted in position 1 by phosphoric acid, this ester would be formed directly by the phosphorolytic cleavage of terminal glucose units from the non-aldehydic end of α-glucopyranoside chain-molecules.

This conception that a direct phosphorolysis of saccharides underlies the formation of glucose-1-phosphate finds support from a number of observations made on the phosphorylase of pea seeds which will be summarized later.

(c) *The effect of alterations of conditions of reaction upon the phosphorylating system*

(i) *Hydrogen-ion concentration.*

The pH-activity relationship of the phosphorylation process has been found to vary, depending upon the concentration of inorganic phosphate. This is shown in figure 3, in which are plotted data from an experiment with a relatively low phosphate concentration—A, and one with a high concentration—B. The digests in the former contained 5 ml. dialysed extract, 60 mg. soluble starch, and, initially, 6·8 mg. *free-P* per 20 ml. (i.e. 0·011 M phosphate). The progress of esterification was followed by method I. Under these conditions, owing to a relatively rapid falling off in rate, an evaluation of the initial velocity is difficult; accordingly, the values for esterified-P after different reaction intervals are plotted against the initial pH of the digests. The initial pH values ranged from 6·1 to 7·8;

these were found to shift slightly to the acid side in the course of 24 hr. reaction in digests having an initial pH of 7 or more, but these changes were insignificant for the present purposes.

The data shown in figure 3 B were obtained in a series of digests in which the initial phosphate was 0·1 M. In these a kaolin-cleared dialysed enzyme (10 ml.) was acting on 36 mg. of soluble starch in digests of 20 ml. Under these conditions the esterification proceeded at an approximately constant rate for an initial period of 1–2 hr. permitting a fairly precise evaluation of the initial velocity.

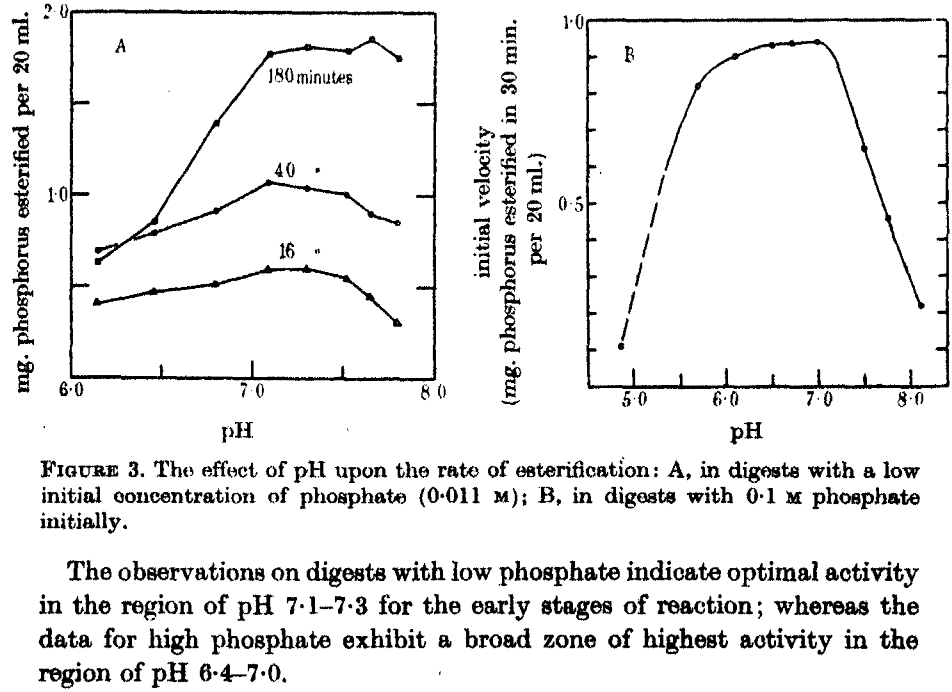

FIGURE 3. The effect of pH upon the rate of esterification: A, in digests with a low initial concentration of phosphate (0·011 M); B, in digests with 0·1 M phosphate initially.

The observations on digests with low phosphate indicate optimal activity in the region of pH 7·1–7·3 for the early stages of reaction; whereas the data for high phosphate exhibit a broad zone of highest activity in the region of pH 6·4–7·0.

The probable explanation of this dependence of the pH optimum upon the concentration of phosphate is that with low phosphate concentrations the conversion of glucose-1-phosphate to hexose-6-phosphates is proceeding rapidly with consequent diminution in the rate of the reaction: glucose-1-phosphate→starch + phosphate. Thus, the observed pH relationship is the resultant of the combined effects of pH upon the phosphorylase and the phosphoglucose conversion system. With high phosphate, however, the phosphoglucose conversion system is strongly inhibited, and the observed effects of pH represent almost exclusively the effects of this factor on the phosphorylase.

(ii) *Concentrations of starch and enzyme.*

In the experiments included in table 3 the effects are shown of variations in a number of reaction conditions.

TABLE 3. EFFECT OF VARYING REACTION CONDITIONS UPON THE ESTERIFICATION PROCESS

Enzyme, dialysed extracts, different preparations used in digests nos. 21–27, 28–29, and 30–31. All values expressed on basis of 20 ml. digest. Esterification followed by method 2.

Digest no.	Soluble starch %	Initial phosphate	Enzyme ml.	mg. P esterified per 20 ml. after the following times (min.)				
				65	180	353	615	1340
21	0·72	0·1 M	8	1·21	2·94	5·30	7·18	8·91
22	0·36	0·1 M	8	1·21	2·89	4·69	5·63	5·67
		7-min.-P/organic-P		—	**0·85**	—	**0·80**	**0·75**
23	0·36	0·2 M	8	0·77	2·22	4·17	5·13	5·96
		7-min.-P/organic-P		—	**0·97**	—	**0·90**	**0·86**
24	0·36	0·1 M	4	0·64	1·74	3·10	4·59	5·79
25	0·12	0·1 M	8	1·07	2·35	2·49	2·50	2·56
26	0·06	0·1 M	8	0·86	1·21	1·37	1·33	1·35
27	—	0·1 M	8	0·01	—	0·11	0·06	0·24
				min.				
				45	90	210	345	1530
28	0·12	0·1 M	8	0·74	1·46	2·45	2·50	2·57
		7-min.-P/organic-P		—	—	**0·97**	**0·95**	**0·68**
29 (as 28 but with 0·006 M $MgCl_2$)				0·74	1·44	2·51	2·54	2·60
		7-min.-P/organic-P		—	—	**0·95**	**0·93**	**0·32**
				min.				
				60	130	200	335	1410
30	0·15	0·125 M	10	1·47	2·29	2·44	2·49	2·55
		7-min.-P/organic-P		—	—	**0·76**	—	**0·31**
				hours				
				5	16·5	24	40	
31	0·75	0·125 M	1·8	1·02	4·70	6·38	8·24	
		7-min.-P/organic-P		**1·01**	**0·98**	**0·99**	**0·99**	

In digests 21–27 the concentrations of soluble starch, phosphate, and enzyme have been varied. No. 27, without added starch, exhibited barely significant increases in *organic-P*.

Comparing nos. 22 and 24 it will be seen that by decreasing the amount of enzyme to one-half, other conditions being constant, the initial rate of the reaction is approximately halved, but the final extent of the conversion is unaltered.

From nos. 22 and 23 it will be seen that increasing the initial concentration of phosphate from 0·1 to 0·2 M inhibits considerably the velocity of the reaction, although the final level of esterified-P was slightly (possibly not significantly) higher. It has been mentioned earlier (cf. also p. 428) that high phosphate concentration strongly inhibits the conversion of glucose-1-phosphate to hexose-6-phosphates; it will be seen from the values *7-min.-P/organic-P* for these digests that the inhibition of the phosphoglucose conversion system was not complete in digests 22 and 23, but that it was greater with 0·2 than 0·1 M phosphate.

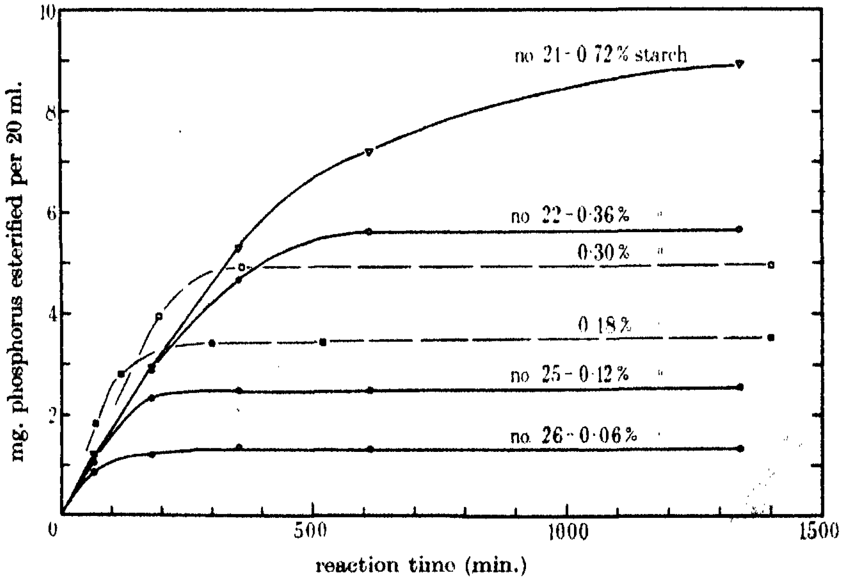

FIGURE 4. The progress of esterification with varying concentrations of soluble starch. Digests 21, 22, 25 and 26 are comparable as regards the enzyme used; the two curves shown in broken lines are from experiments in which different enzyme preparations were used.

In digests 21, 22, 25 and 26 the concentration of soluble starch was varied over a wide range. Curves showing the progress of esterification in these digests are given in figure 4, and in addition two progress curves obtained in other experiments (with different enzyme preparations) in which intermediate concentrations of starch were used.

Although these data do not permit a precise evaluation of the initial reaction velocity, it is evident from figure 4 that this is not appreciably altered by variations in starch concentration from 0·06 to 0·72 %. It is probable, therefore, that the phosphorylase exhibits a relatively high affinity for starch, considerably higher, for example, than the α- and β-amylase from malt.

Typically the progress curves show an initial phase during which the velocity falls only slightly; this is terminated by a phase of rapidly decreasing velocity, and (except in digest 21 with the highest starch concentration) a constant final value was reached in the period under observation.

From these final levels of esterified-P the extents of conversion of the substrate into hexose-monophosphate have been calculated (1 mg. esterified-P ≡ 5·22 mg. anhydrohexose). It is found that the percentage conversion of soluble starch into hexose-monophosphate varies considerably with the concentration in which it is added; for the digests shown in figure 4 the values are:

Initial starch concentration	0·06	0·12	0·18	0·30	0·36	0·72
Final percentage conversion into hexose-monophosphate	58	55	52	43	41	(32)

With increasing concentrations of starch, the proportion converted into hexose-monophosphate diminishes progressively.

The present data do not permit a full analysis of this effect but certain points deserve mention. There is considerable evidence which suggests, for example, that the final conversion value attained for a particular concentration of starch is not related to extent of the transformation of glucose-1-phosphate into hexose-6-phosphates; thus the final conversion value has been found to be remarkably constant with different enzyme preparations in spite of large variations in the activity of the phosphoglucose conversion system; it may be shown, for example, by the addition of Mg^{++} (cf. digests 28 and 29) that the resultant activation of the phosphoglucose conversion system is without effect upon the extent of esterification.

The available data indicate that in digests with high phosphate concentration and small amounts of starch the cessation of esterification is due to the disappearance of suitable carbohydrate substrates for the phosphorylase. The dependence of the conversion limit upon the initial starch concentration under these conditions appears to arise from the competitive action of phosphorylase and amylase on the available substrates (starch and dextrins). Whereas the complex dextrins formed by the action of the amylase are readily acted upon by the phosphorylase, it was made clear earlier that simpler dextrins are esterified to a lesser extent, and that glucose (which is formed in considerable amount by the amylase) is not acted upon at all. Thus if the amylase exhibits a higher affinity for the substrate than the phosphorylase, an effect of the type observed would be expected to occur. On the other hand, when relatively high concentrations of starch are added, the esterification proceeds until a state of equilibrium (governed by the relative concentrations of inorganic phosphate and glucose-1-phosphate) is attained; this will be made clear in a subsequent communication.

Finally, in connexion with the data given in table 3, attention is drawn to a feature illustrated in digests 30 and 31, which has been observed repeatedly.

It is found that the degree of dilution of the enzymic extract added to digests with high phosphate concentration affects differentially the formation of glucose-1-phosphate, on the one hand, and the subsequent conversion of this product into hexose-6-phosphates, on the other. When a relatively small volume of enzyme is added (e.g. $\frac{1}{10}$ to $\frac{1}{20}$ of the total volume of digest), the rate of action of the phosphorylase is approximately that expected on the basis of a linear relationship between enzyme concentration and reaction velocity, whereas this appears not to hold in the case of the phosphoglucose conversion system. For example, in digest 30, to which a fresh preparation of a kaolin-treated, dialysed enzyme was added in the proportion of 10 ml. in a 20 ml. digest, the conversion of glucose-1-phosphate to the hexose-6-phosphates had proceeded to an appreciable extent in 200 min. In digest 31, carried out simultaneously, the same extract was added at the rate of 1·8 ml. in 20 ml. digest, and here it will be seen the ratio *7-min.-P/organic-P* did not fall significantly during a prolonged reaction period.

While it may be suspected that this effect is due to the dilution of a co-enzyme of the phosphoglucose conversion system (e.g. Mg^{++}) which may be present in very low concentration in dialysed extracts, no direct experimental evidence is yet available. As will be seen in the following section, however, use has been made of this effect in large-scale digests carried out for the preparation of glucose-1-phosphate.

(*d*) *The isolation of the hexose-phosphates formed from starch by the action of extracts of peas*

Observations will now be described on three digests (nos. 31–33), in which the reaction conditions were adjusted so as to result in the accumulation of the different hexose-phosphates which represent successive stages in the transformation of starch to hexose-diphosphate.

(i) *The formation and isolation of glucose-1-phosphate.*

In digest 31, of which the final volume was 10 litres, 900 ml. of kaolin-cleared dialysed extract was allowed to act upon 75 g. (dry basis) of soluble starch at pH 6·8. The initial concentration of phosphate was 0·125 M.

The progress of esterification was followed by method 2 and the observations have already been given in table 3, no. 31 (where for convenience they were expressed on the basis of 20 ml. digest).

After 40 hr. reaction the analyses indicated the esterification of 4·22 g. P per 10 l., of which 99 % was in the form of 7-*min.-P*. This would correspond with the formation of 50·2 g. of the crystalline potassium salt of glucose-1-phosphate (in which form the ester was isolated).

At this time the action was arrested by the addition of 50 ml. 1 % iodine solution, and the digest was concentrated by a process of removal of water by freezing. This was accomplished by allowing it to stand at − 10° C with occasional stirring to reduce the masses of ice crystals to an even slush. The liquid was separated from the ice crystals on a centrifugal machine (fitted with a perforated basket), and in order to wash the ice, this was allowed to thaw slightly, and the resultant liquid again separated. By this means the digest liquor was concentrated to 2 l. without loss of 7-*min.-P*.

The inorganic phosphate was now removed by the addition of 1·25 g.mol. of magnesium acetate followed by ammonium hydroxide in slight excess of the calculated amount. After 2 hr. the precipitated phosphate was filtered off; the residue was washed by suspending in 300 ml. water and again filtering. The washing was recombined with the main filtrate. To this solution was added 35 g. barium acetate (dissolved in 100 ml. water) followed by 2 vol. of 95 % alcohol. After 15 hr. the precipitate of the crude barium salt was removed by centrifuging and, without drying, was suspended in 600 ml. water.

The next treatment was evolved as the simplest means of removing the impurities associated with the barium salt, namely, dextrins and protein. The suspension was acidified to pH 1·0 by the addition of 2 N sulphuric acid and then reneutralized to pH 4·0 with 2 N potassium hydroxide. After standing for 2 hr. with occasional stirring, 26 g. powdered trichloracetic acid was added and dissolved by stirring. Two volumes of 95 % alcohol were now added, and after half an hour the precipitate, consisting of barium sulphate, protein and dextrin, was removed on the centrifuge. The precipitate was washed by suspending in 200 ml. 80 % alcohol, and after centrifuging the supernatant was combined with the main liquor.

The acid alcoholic solution (amounting now to a volume of about 2 l. and containing approximately 60 % alcohol) was brought to faint alkalinity by the addition of strong potassium hydroxide (96 ml. of 25 % solution required). Crystallization of the potassium salt of glucose-1-phosphate began almost immediately, and this was allowed to proceed overnight at 0° C. The crystalline product was collected and dried to constant weight *in vacuo* over calcium chloride. The yield was 43 g. representing 86 % of that calculated from the analyses on the original digest.

In this particular experiment no attempt was made to recover the further small amounts of ester present in the different mother liquors and precipitates, but this has been done in other smaller scale digests, and in one case a yield of 93 % of the crystalline product was obtained.

The observations on digest 31 have been chosen for description in that the procedure adopted in this case represents the simplest method that could be devised for obtaining a reasonably high yield of the product in a state of high purity. This procedure embodies certain features which were used by Kiessling (1938) for the isolation of this same product from digests of glycogen by muscle extracts, and it should be stated that, as a preliminary to any attempt to isolate the product from digests by the pea system, this preparation with muscle extract was repeated carefully with results in agreement with Kiessling's observations. The considerable modifications in procedure which have been introduced are necessitated by the different character of the accompanying impurities and by the larger scale of the experiments.

After its first crystallization the product, as obtained in digest 31, consists to the extent of 97–98 % of the dipotassium salt of glucose-1-phosphate (with two molecules of water of crystallization). Recrystallization was normally carried out by dissolving the salt in 20 times its weight of water and adding an equal volume of absolute alcohol. As before, crystallization was allowed to proceed for a few hours at room temperature and then overnight at 0°.

The following observations were made on a recrystallized specimen:

Moisture content. Crystals, first dried to constant weight *in vacuo* over calcium chloride, lost 9·68 % of their weight at 110° *in vacuo* (with P_2O_5 in a side vessel). $C_6H_{11}O_5.PO_4K_2.2H_2O$ requires 9·64 % water.

Drying to constant weight at 56° *in vacuo* (1 mm.) removed only 1 mol. of water (observed loss 4·95 %; calculated 4·82 %) and only a small further loss (0·40 %) occurred during 3 hr. at 80°; the second molecule of water was removed rapidly at 100°.

Rotation. $[\alpha]_D^{21^\circ}$ + 76·7° for crystalline salt (c = 1·123), + 85° for anhydrous salt, dried at 100° *in vacuo* (c = 0·716).

These values are in exact agreement with those found for a recrystallized specimen of the dipotassium salt moisture content 9·65 %, isolated by Kiessling's method from a digest of glycogen by muscle extract.

Phosphorus. Apparent *free-P*, after 20 min. contact with 0·77 N perchloric acid contained in the reagent, 0·21 %; after 40 min. contact, 0·32 %.

Total-P, after combustion, 8·35 %.

7-min.-P, 8·32 %.

Calculated for $C_6H_{11}O_5.PO_4K_2.2H_2O$, 8·31 %.

Products of hydrolysis. The reducing power of the recrystallized salt was less than 0·1 % that of glucose (amounts up to 10 mg. showed no reducing power to an alkaline copper reagent under conditions in which 0·01 mg. glucose was detectable).

5 ml. of a 4·00 % solution of the crystalline hydrate were heated for 7 min. at 100° with 1 ml. 6 N HCl. This was neutralized and made to 25 ml. The reducing power of this solution was now found to be equivalent to 3·82 mg. glucose per ml. and the rotation ($l = 2$ dm.) was $+0·41°$ (calculated for glucose liberated from $C_6H_{11}O_5.PO_4K_2.2H_2O$, glucose 3·86 mg. per ml.; $r = +0·415°$).

Further investigations now in progress are aimed at the establishment of the configuration of this ester; meanwhile the above observations, together with its behaviour in a variety of enzyme systems, serve to establish its identity with the glucose-1-phosphate described by Cori and Cori (1936) and Kiessling (1938).

(ii) *The formation and isolation of hexose-6-phosphates.*

In digest 32, of which the initial volume was 2·1, 200 ml. of a dialysed extract (corresponding to 48 g. original pea flour) was allowed to act on 25 g. soluble starch at pH 7·5. The initial concentration of phosphate was 0·01 M (≡ 620 mg. P in all) and 0·002 M magnesium chloride was also added. After 15 hr. reaction a further addition of phosphate equal to the amount present initially was added.

After 43 hr. reaction at 25°, protein was precipitated by the addition of trichloracetic acid to give a concentration of 5 %. Analyses of the filtrate showed it to contain 309 mg. *free-P*, 1005 mg. *organic-P*, of which 58 mg. reacted as *7-min.-P*. Thus 75 % of the added inorganic phosphate had been esterified. Of the 1005 mg. *organic-P*, 57 mg. were accountable by the acid-soluble *organic-P* which was found to be present in the enzyme added to the digest. Thus the increase in *organic-P* amounted to 948 mg.

The acid filtrate was brought to pH 6·5 with 10 % sodium hydroxide and 75 ml. 40 % barium acetate were added. A slight precipitate formed which contained mainly inorganic phosphate. Alcohol to give a concentration of 10 % was added and the pH was adjusted to 8·0. Only a trace of precipitate was formed and it was found that only 50 mg. *organic-P* was removed during these two operations, indicating that no significant amounts of hexose-diphosphate were present.

Alcohol was added to give a concentration of 70 % and the copious precipitate which resulted was collected and dried. This product (20·1 g. dry weight; 3·98 % *organic-P*) contained 89 % of the esterified-P.

The separation of the hexose-phosphates from this material, the principal contaminant of which was dextrin, occasioned considerable difficulty. The most successful method of removing the dextrin was to dissolve the crude product in water, discarding a small amount of insoluble material, and to adjust the solution to pH 2–2·5 with sulphuric acid; then, after removing barium sulphate, 3 vol. of 95 % alcohol were added, whereby a syrupy precipitate of dextrins was formed. The latter material contained appreciable quantities of *organic-P* and it is not possible as yet to state whether or not this was due exclusively to the occlusion of hexose-phosphates during the precipitation, although this is thought to be probable. The main portion of the *organic-P* remained in the acid alcoholic supernatant liquor, from which it was recovered, together with a small amount of barium sulphate, by the addition of barium acetate, the solution being first brought to a faintly alkaline reaction with sodium hydroxide. The barium sulphate was then eliminated by dissolving the product in water, centrifuging, and reprecipitating the dissolved material with alcohol.

After a single application of this treatment to a portion of the crude material, a product containing 70 % of the original *organic-P* was obtained which had the following properties: Ba content, 33 %; *organic-P*, 6·40 %; $[\alpha]_D^{20^\circ} + 13{\cdot}7^\circ$.

A repetition of this acid-alcohol treatment was followed by two precipitations of the barium salt from neutral solution, and then treatment in aqueous solution with a small amount of blood charcoal (at room temperature). The barium salt, which was then precipitated with alcohol and dried, was found to give analytical values approaching those for the barium salt of a hexose-monophosphate.

The following values expressed on the basis of anhydrous material were obtained on analysis:

Ba content, 36 %; *organic-P*, 7·1 %; *free-P*, 0·05 %.

Specific rotation, $[\alpha]_D^{21^\circ} + 12^\circ$ ($c = 0{\cdot}7$; $l = 4$ dm.).

Reducing power (glucose = 100): (*a*) by copper reagent (Somogyi 1937), 30·5; (*b*) by hypoiodite (Macleod and Robison 1929), 25·0.

Apparent fructose (by a modified HCl-resorcinol method), 6·7.

Hydrolysis of *organic-P* at 100° in 1 N HCl ($c = 0{\cdot}32$ mg. *organic-P* per ml.). 120 min., 25 %, 180 min., 29 % hydrolysed.

These data establish the close similarity of the product with the mixture of barium salts of hexose-6-phosphates isolated by Robison (1922) from a yeast preparation.

In the present experiment the product as analysed contained approximately 60 % of the phosphorus which was esterified in digest 32; the possibility is accordingly not excluded that other esters may have been formed. Further investigations of the reducing hexose-monophosphates which are formed from glucose-1-phosphate by the action of dialysed extracts are in progress.

(iii) *The formation and isolation of hexose-diphosphate.*

The isolation of hexose-diphosphate requires only brief mention since this constitutes merely a confirmation of the findings of Tankó (1936). The conditions of reaction differed from those adopted by Tankó in that a smaller amount of pea flour (in suspension) formed the source of the enzyme and that soluble starch was added as substrate.

Thus in digest 33, of which the final volume was 1·5 l. and the pH 7·5, 37 g. pea flour, 18 g. soluble starch, and 0·01 M phosphate were added initially. After 18 and 36 hr. further equal additions of phosphate were made.

The total inorganic phosphate, including that present in the pea flour, was equivalent to 1425 mg. *free-P*. Of this, 1010 mg. was esterified during 48 hr. action. At this time proteins were precipitated with trichloracetic acid and the filtrate was found to contain 1450 mg. *organic-P*, of which a considerable part must have been derived from phosphorus-containing compounds in the pea flour.

Attention was directed solely to the fraction of which the barium salts were insoluble in 10 % alcohol. The trichloracetic acid filtrate was neutralized and the precipitate, which consisted largely of phytin, was removed and discarded. Barium acetate and alcohol to 10 % concentration were then added to the clear solution. The resultant precipitate was suspended in water, the acidity was adjusted to pH 3·0 and a small amount of insoluble material was removed. The solution was next adjusted to pH 4·0 and the crude acid barium salt was thrown down by the addition of 4 vol. of alcohol. This product (9·8 g. dry matter) contained 730 mg. *organic-P*.

A repetition of the precipitation from acid alcohol failed to effect any considerable purification and the product was then converted into the neutral barium salt. Analysis showed it to be still impure, the main contaminant being apparently barium phosphate (*organic-P*, 7·2 %; *free-P*, 2·1 %). Observations on the rate of hydrolysis of the esterified phosphate present in this fraction showed a close agreement with the values reported for pure fructofuranose-1 : 6-diphosphate. These observations, which are given in table 4 together with the nature of the procedure used in isolating the fraction, indicate clearly that the esterified phosphate was in the form of the fructofuranose-1 : 6-diphosphate.

TABLE 4. HYDROLYSIS IN 1 N HCl AT 100° OF HEXOSE-DIPHOSPHATE

A, product from digest no. 33 (0·447 mg. *organic-P* per ml.). B, data from Macleod and Robison (1933) for pure fructofuranose-1 : 6-diphosphate (0·454 mg. *organic-P* per ml.).

	Time of heating (min.)						
	5	10	25	60	120	180	300
A. Percentage hydrolysis of *organic-P*	22·0	34·5	49·5	67·7	79·9	90·5	95·0
B. Percentage hydrolysis of *organic-P*	23·3	36·8	54·4	68·8	83·5	90·2	96·6

A closer characterization of specimens formed by the action of purified enzyme preparations on hexose-monophosphates will be described later.

(*e*) *The synthesis of starch from glucose-*1*-phosphate*

The reversibility of the first step in the chain of reactions surveyed above is readily demonstrated by a simple experiment with a filtered, aqueous extract from ground peas. When a small amount (e.g. 3–10 mg.) of the potassium salt of glucose-1-phosphate is added to 2–3 ml. of such an extract, the synthesis of polysaccharide soon becomes evident if portions of the solution are tested at intervals with dilute iodine. Within a few minutes a mauve colour is given on the addition of iodine. Subsequent samples exhibit a purple iodine colour and this rapidly becomes more intense as the reaction proceeds. Later, for example after one hour, a red-violet colour is observed, and symptomatic of a progressive degradation of the polysaccharide this becomes rose, orange and finally no coloration is developed.

An investigation of the reactions underlying this effect has revealed that the addition of glucose-1-phosphate to a crude extract of pea flour initiates a complex set of processes. The following main reactions are recognizable:

(1) Glucose-1-phosphate is transformed progressively, and apparently irreversibly, into reducing hexose-6-phosphates.

(2) After a short induction period, glucose-1-phosphate is transformed into a polysaccharide, resembling starch or a complex starch-dextrin exhibiting a purple iodine colour, and inorganic phosphate is liberated.

(3) The polysaccharide (formed in reaction 2) is progressively hydrolysed by the action of amylase present, with the formation of dextrins of varying complexity.

(4) Hexose-6-phosphate (formed in reaction 1) is transformed slowly into hexose-diphosphate by esterification at the expense of inorganic phosphate (present initially in the extract and formed in reaction 2).

(5) When the concentration of glucose-1-phosphate falls to a certain level (as a result of reactions 1 and 2) reaction 2 ceases, and on further reduction in the amount of this ester (by the continuance of reaction 1) the reversal of reaction 2 sets in; that is, the re-conversion into glucose-1-phosphate of the polysaccharide previously formed in reaction 2, and dextrins formed from it (in reaction 3).

By varying the conditions of reaction and by different preliminary treatments of the extract it has been possible to alter enormously the relative velocities of these different reactions. For example, at pH 8–8·5 reaction 1 proceeds rapidly while the velocity of reaction 2 is negligibly small; at about pH 6·4 the velocity of reaction 2, relative to that of reaction 1, is maximal. Again on storing dialysed extracts (without kaolin-treatment) at 2° C, the activity of the phosphoglucose conversion system falls much more rapidly than that of the phosphorylase; after 8–15 days' storage, several extracts were found to be devoid of the former. In these cases, only reactions 2 and 3 occurred on the addition of glucose-1-phosphate, and it was found that reaction 2 proceeded until 60–65 % of added ester was transformed into polysaccharide and inorganic phosphate, the ratio *free-P/free-P + 7-min.-P* attaining a steady value of about 0·8. It was possible by pre-treating such extracts with low concentrations of iodine, or copper sulphate, to inhibit the amylase almost completely while a somewhat feeble phosphorylase activity remained; in such extracts, reaction 2 proceeded slowly, with a virtual absence of reaction 3, and it was observed under these conditions that the colour given with iodine was initially a faint green; this changed to a brilliant blue, which in increasing intensity persisted for prolonged periods (e.g. 24 hr.) before indications of degradation of the polysaccharide became apparent. Observations on such extracts indicated that the final level of conversion attained is but little influenced by the presence or absence of amylase, but is markedly influenced by the concentration of inorganic phosphate.

While such experiments have served to establish the general character of the reactions which occur, it became evident that for a detailed analysis of the equilibrium between glucose-1-phosphate, on the one hand, and starch and inorganic phosphate, on the other, it was essential that the phosphorylase should be isolated in an active form, free from both the amylase and the phosphoglucose conversion system. This object has been achieved recently and the investigation of the system is in progress. One striking fact which has emerged in preliminary experiments with these preparations is that the polysaccharide is actually deposited from solution in the form of small rounded granules, which stain a deep blue colour with iodine. These granules which are often surrounded by a light flocculent material, apparently protein in nature, appear in the clear enzyme solution during the course of the reaction.

CONCLUSIONS

The foregoing experiments constitute a preliminary survey of a system of enzymes from pea seeds which, in the presence of inorganic phosphate, catalyses the formation of hexose-phosphates from starch, various dextrins, and maltose.

The first recognizable step in the process is the formation of glucose-1-phosphate which, under suitable conditions, accumulates as the sole product of the process of esterification. The isolation of this ester in the form of the crystalline potassium salt has been described. It appears to be identical with the glucose-1-phosphate which is formed as the primary product of esterification during the action of the phosphorylase of muscle on glycogen.

A number of observations support the conception which was advanced by Parnas (1937) in connexion with the phosphorylase of muscle, that glucose-1-phosphate arises by a process of direct phosphorolysis of the saccharide chains of the substrate.

Thus, although the phosphorylase from peas acts upon a wide range of saccharides of the starch-maltose series, it has been shown that the more complex molecules are esterified more rapidly and to a greater final extent than are simpler dextrins or maltose. Evidence that starch and complex iodine-staining dextrins participate directly in the reaction is afforded by the demonstration that the action of the phosphorylase results *per se* in a decrease in the iodine colour. Furthermore, the change in the iodine-colouring property of the substrate is of the type which would be expected to occur during an end-wise degradation of the chain molecules. These observations, together with the fact that glucose is not esterified, are thus in harmony with the hypothesis that the α-glucose chains are degraded by the phosphorolytic cleavage of terminal glucose units, these being liberated successively (from the non-aldehydic chain-ends) in the form of glucose-1-phosphate.

The transformation of starch into glucose-1-phosphate has been found to be a reversible reaction. Thus when glucose-1-phosphate is added to an extract from peas it becomes partially converted into a polysaccharide which resembles starch, and inorganic phosphate is liberated. A detailed analysis of this reaction has been deferred until further data, obtained with more highly purified preparations of the phosphorylase, are available.

In an alternative reaction, glucose-1-phosphate is converted into reducing hexose-monophosphates, of which a considerable proportion has been shown to consist of 6-phosphoric esters of glucose and fructose. This transformation is catalysed by two or more enzymes which have been referred to as the phosphoglucose conversion system.

Finally, when crude extracts of peas are allowed to act upon starch, in the presence of inorganic phosphate, fructofuranose-1 : 6-diphosphate is formed. This ester is not formed, however, when dialysed extracts are used. It seems likely that a dialysable co-enzyme is required by the mechanism concerned in this phosphorylation but the question requires further investigation.

It is too early as yet to attempt any detailed evaluation of the part played in the metabolism of plants by enzymic mechanisms of the type disclosed in this preliminary study. There seems little doubt, however, that the particular interconversions of carbohydrate forms, which have been shown to occur so readily under the action of the enzyme system of peas, will be found to throw light on the known peculiarities of the so-called "starch-sugar" balance in plants and on the production of the substrate of glycolysis.

I wish to thank my colleague, Dr R. J. L. Allen, for his advice throughout the investigation and for his criticisms of the manuscript. I am indebted to Mr E. T. Whitmore and to Mr W. T. Spragg for most valuable help in the conduct of the experiments. Finally I wish to thank Dr F. F. Blackman, F.R.S., for the stimulating interest he has shown in this work.

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SUMMARY

An investigation of a system of enzymes from pea seeds which catalyses the formation of hexose-phosphates from starch, various dextrins, and maltose is described.

The first recognizable step in the process is the formation of glucose-1-phosphate which has been isolated in the form of the crystalline potassium salt. Evidence is advanced supporting the conception that this ester is formed as the result of a process of direct phosphorolytic cleavage of terminal glucose units from the non-aldehydic ends of the chain molecules of the substrate.

This primary conversion of starch into glucose-1-phosphate is shown to be a reversible reaction, catalysed in both directions by an enzyme which has been termed phosphorylase.

Glucose-1-phosphate takes part in an alternative reaction, catalysed by the phosphoglucose conversion system, in which it is converted into reducing hexose-monophosphates.

Finally, in non-dialysed extracts, fructose-diphosphate is formed.

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Host selection by hymenopterous parasites of the moth *Plutella maculipennis* Curtis

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[Plates 18 and 19]

1. Introduction

Criticism of the procedure of earlier exponents of biological control in introducing all primary parasites and predators of a given pest, has usually stressed the harm which might result from directly competing species. It has been suggested that a more cautious approach should be made in the form of introduction of the parasitic or predacious species singly, later additions to follow if observation indicates the necessity (Thompson 1930; Sweetman 1936). While this method may be prudent, the usually slow nature of biological control work results in its rejection for financial reasons. There is, perhaps, some justification for this attitude, when one recalls instances such as that of the Hessian fly parasite, *Pleurotropis metallicus*, which, although introduced from England into the U.S.A. in 1894, was not recovered until twenty-one years later. It has since become one of the dominant parasites of this host in the eastern states.

The thesis of selection from a group of primary parasites of a given host pest has been stressed by various authors, particularly Taylor (1937) and the Hawaiian workers, Pemberton, Willard and Bissell (1918, etc.). The theory has been criticized by H. S. Smith (1929) on the ground that slight multiparasitism and competition are more than counterbalanced by the diverse ecological requirements of several different species of parasites. Smith's criticism would appear to be borne out by later results in the Hawaiian fruit fly investigations of Willard and Mason (1937). This latter paper reviews the work from 1914 to 1933 and does not repeat the claim of earlier papers that it was a mistake to introduce some of the Opiine species.

It is generally conceded that when the primary parasites attack different stages of the host, no ill-effects will result from the introduction of all species. The problem of selection arises when two or more species of primaries attack the same or similar stages of the host, with consequent competition.

In this respect the establishment in Hawaii of the four species of parasites, all of which attack the host larva when it is more than half-grown, seems a rather exceptional case on which to generalize; the more typical condition is that of a host attacked by a sequence of parasites with the result that interparasitic competition is reduced in importance (Tothill 1922). The fact that with the Hawaiian fruit fly, *Ceratitis capitata*, the braconid parasites *Opius humilis* Silvestri and *O. fullawayi* Silvestri were obtained from South Africa and *O. tryoni* Cameron from Australia, and that this latter species is said to exercise a controlling influence over the more prolific *O. humilis*, possibly suggests that caution should be observed before introducing parasite species attacking the same host stage, but occurring naturally in widely separated ecological areas. Such a principle cannot be established on the basis of one experiment.

Apart from a brief notice by Taylor (1937) in his work on the parasites of the leaf mining beetle *Promecotheca reichei* in Fiji, few data are available on this topic of possible competition between proposed introductions, and the investigation by Farnham House Laboratory of the European parasite complex of the diamond back moth, *Plutella maculipennis*, seemed to offer a good opportunity for extending knowledge of this aspect of biological control work.

2. METHOD

Examination of the literature and extensive surveys in Europe by the Laboratory staff and Mr J. Muggeridge, New Zealand Government Entomologist, have indicated that the more common European parasites of *Plutella maculipennis* are: the ichneumonids *Angitia cerophaga* Gravenhorst, *A. fenestralis* Holmgren, and the braconid *Apanteles plutellae* Kurdjumov, attacking the larvae; the ichneumonids *Diadromus* (*Thyraeella*) *collaris* Gravenhorst and *D. subtilicornis* Gravenhorst attacking the prepupae and pupae. All species are internal parasites. It is proposed to investigate the incidence of multi- and superparasitism in these parasites in the field and laboratory. The subject-matter of the present preliminary paper is mainly the laboratory experimental work on the three species, *Diadromus collaris*, *Angitia cerophaga* and *Apanteles plutellae*. A few data on field parasitism by these species are included. Throughout the work the terms superparasitism and multiparasitism are used in the sense defined by Smith (1929). Multiparasitism (or multiple parasitism) involves two or more individuals of different species in a host; superparasitism two or more specimens of the same species.

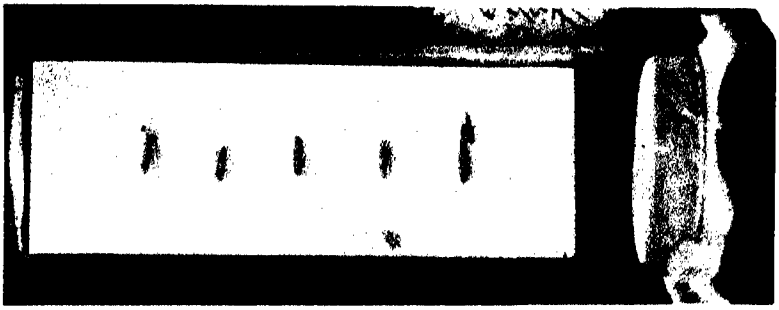

FIGURE 1. Method of exposure of *Plutella* pupae to *Diadromus collaris* (natural size).

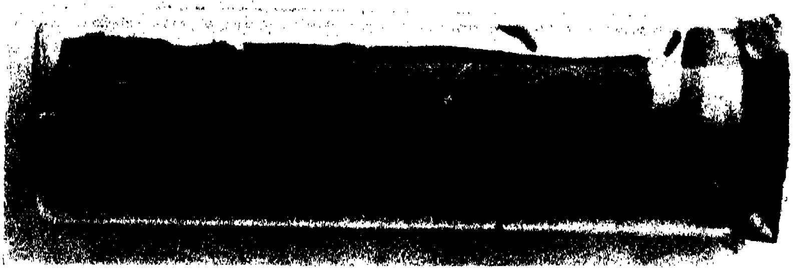

FIGURE 2. Method of exposure of *Plutella* larvae to *Angitia cerophaga* (natural size).

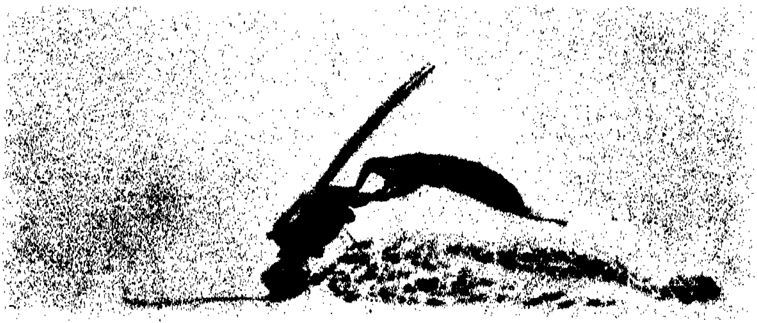

FIGURE 3. Female of *Diadromus collaris* feeding on *Plutella* pupa (× 36).

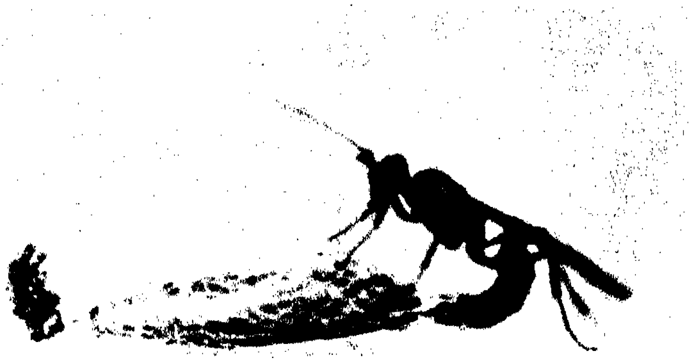

FIGURE 4. Female of *Diadromus collaris* releasing ovipositor from sheaths (× 36).

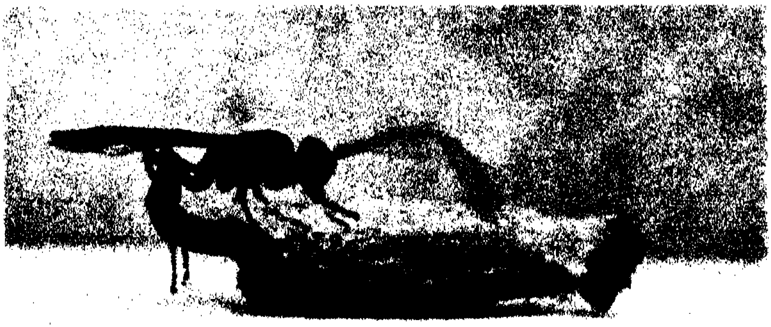

FIGURE 5. Female of *Diadromus collaris* with ovipositor fully inserted in host pupa.

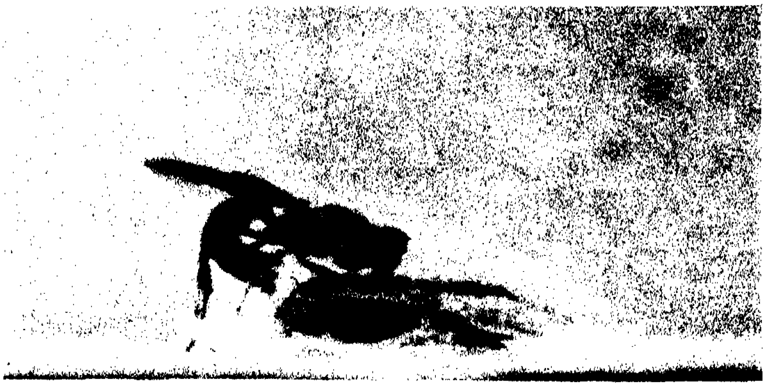

FIGURE 6. Female of *Diadromus collaris* inserting ovipositor while her head and thorax are enclosed by the host cocoon.

(Photographs by Mr H. S. Hanson)

From the experimental point of view, the problem of multiple parasitism is being regarded as a question of the nature of the oviposition responses of the various parasites. The procedure adopted consists in confining the female parasites in tubes (4 in. long, 1 in. diam.) for a predetermined time with a given number of hosts and observing the oviposition behaviour of the adult parasites and subsequent development of the immature parasites to note the effects of direct competition. Prepupae and pupae are lightly gummed by their cocoons on a strip of stiff drawing paper; in the case of host larvae, a strip of cabbage is so wedged in the tube that all larvae are as accessible to the female as the experimenter can arrange (figures 1, 2, plate 18). The tubes are closed by perforated corks surrounded with fine muslin. All experiments are performed under constant temperature and humidity. It is realized, of course, that this method has many limitations. Oviposition in the field probably depends upon such factors as the density and accessibility of the host, density of the parasites, etc., so that it is clear that the prevalence of multiple parasitism in any particular country will depend upon, and can only be accurately assessed in, the conditions prevailing in that country. Nevertheless, laboratory work does indicate potentialities, and it is thought that the method adopted does give some idea of the host conditions in which the various females will oviposit and hence some of the predisposing factors for multiparasitism. It is hoped, too, that the investigation will furnish data on the relation between behaviour in the laboratory and field.

3. OVIPOSITION BEHAVIOUR OF THE PARASITES

(*a*) *Diadromus* (*Thyraeella*) *collaris* Grav.

The females attack and oviposit in a wide range of *Plutella* prepupae* and pupae—even the prepupae immediately after the spinning of the silken cocoons by the mature larvae. Oviposition is limited only when the imaginal organs are well advanced in the pupa and the contents of the latter have lost the fluid consistency of the earlier stages.

The presence of the silken cocoon surrounding the pupa plays an appreciable part in the acceptance and parasitization of a particular host. This will be seen from table 1.

In experiment A, ten fertilized females were each supplied daily with ten host pupae removed from the cocoons; on the eighth and subsequent days they were given normal pupae enclosed in cocoons. In experiment B, another ten females were supplied with pupae enclosed in cocoons for 7 days prior to being given access to naked pupae. The females in this latter experiment were of various ages, so that oviposition was irregular. The immediate increase or decrease in laying when the cocoon is present or absent is apparent. A few experiments were done in which naked pupae were placed alongside empty silken cocoons but oviposition was still very limited. A somewhat similar observation on the importance of the silken cocoon in securing oviposition has been noted by Jackson (1937) with *Pimpla examinator* Fabricius. Some of the females used in experiments with the Pimpline would not oviposit in the naked pupae of various Lepidoptera.

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* The prepupal stage is taken as commencing immediately after cocoon formation.

TABLE 1. THE EFFECT OF COCOON REMOVAL ON THE OVIPOSITION OF *DIADROMUS COLLARIS*

	Eggs laid on day no.									
	1	2	3	4	5	6	7	8	9	10
Exp. A	0	9	11	23	22	23	31	*57*	*69*	*85*
B	39	42	63	63	59	65	53	*9*	*12*	—

The host cocoon is of significance in the oviposition response apparently because of the peculiar posture adopted by the female in the act of oviposition. This silken cocoon surrounding the host is so spun that apertures are left in the region of the head and tail ends of the pupa. Innumerable observations of females attacking the hosts have demonstrated that the ovipositor must be inserted at one of these points (in the manner of figures 4, 5, plate 19). On encountering a host the female mounts the cocoon at one end and walks to the other end, subjecting the cocoon to incessant antennal examination. If this examination is satisfactory, the female, while poised at one end of the cocoon, turns through an angle of 180° and inserts the ovipositor at the aperture last examined by the antennae. The head and thorax of the adult parasite now lie over the upper surface of the cocoon, while the abdomen is bent more or less parallel to the thorax and with its ventral surface facing away from the host surface. The tip of the abdomen is then flexed so as to make contact with the host and the ovipositor is released from its sheaths and driven into the pupa. In so doing the tip of the abdomen is forced backwards until the ovipositor is inserted up to the base. The duration of the action varies from 30 sec. to 4 min., the variation being due to difficulties in securing the insertion of the ovipositor through the incomplete ends of the cocoons and the release of the ovipositor from its sheaths.

There seems to be some preference for the head region of the pupa, and in one observed series of fifty-two attacks, forty were in the head. The aperture at the end of the cocoon near the pupal head is usually better defined than that at the tail end, where it is frequently obscured by the presence of the cast skin of the mature host larva (figure 5, plate 19). Females have never been seen to attempt a direct penetration of the meshes of the upper surface of the rather loosely woven cocoon, and even when the cocoon is removed the parasites flex the abdomen in the normal manner. Apparently in the absence of a cocoon difficulties occur in securing the release of the ovipositor from the sheaths as the abdomen is able to move too freely. The inflexibility of the oviposition response from this aspect has been demonstrated on several occasions when females have been observed examining a cocoon from which the host has been partially dislodged by enlargement of one of the terminal holes so that the pupa lies partly to one side of the cocoon. In such cases the females still persist in inserting the ovipositor into the empty portion of the cocoon enclosure although the pupa lies exposed nearby. When there is a very large aperture at one end a female sometimes enters the cocoon and attempts insertion of the ovipositor (figure 6, plate 19).

When there is opportunity the *Diadromus* females show a distinct preference for the younger prepupal and pupal stages, but oviposition does not seem to be appreciably restricted when no choice is available. This will be seen on examination of tables 2 and 3. Ten females were used in both experiments and each was supplied with ten hosts daily throughout life. In one series the ten parasites were given a choice of prepupae and pupae and these host types were alternated at half-inch intervals on paper. The experiments were performed at a constant temperature of 20° C, at which temperature the host larvae spin up and remain as prepupae for 24–36 hr. The pupae in the experiments were obtained by waiting for the pupation of such collected prepupae. A further experiment at 20° C, with six fertilized females given a choice of prepupae and 4-day-old pupae over several days gave 175 eggs in the prepupae and sixty-two in the 4-day-old pupae.

It will be noticed that there was a very heavy superparasitism under the conditions prevailing in the foregoing experiments, and as only one parasite is able to mature in a host this results in appreciable mortality. These data suggest that the females were unable to distinguish between parasitized and unparasitized hosts. Ten hosts of an apparently uniform nature were supplied to each female daily and although at certain times of a female's life more than ten eggs were laid in a day, it was exceedingly rare to find any female utilizing all ten pupae in 1 day.

This heavy superparasitism was not the result of the removal of the pupae from the cabbage leaves. This operation sometimes slightly damages the cocoons but does not seem to render some hosts more accessible than others, as superparasitism was of the same degree if the hosts were left on the host plant. Moreover, superparasitism was as prevalent in pupae which had been removed from the cocoons. For example, one series of ten females, supplied with ten such hosts daily, laid 119 eggs in eighty-seven hosts during the first week of life: comparable figures extracted from tables giving the oviposition in normal pupae gave 119 eggs deposited in 104 hosts of the 700 available over a period of 7 days.

TABLE 2. OVIPOSITION OF *DIADROMUS COLLARIS* IN *PLUTELLA* PREPUPAE AND PUPAE

	Females										
	A	B	C	D	E	F	G	H	I	J	Totals
Length of life (days)	68	58	60	18	46	50	62	81	43	43	529
No. of hosts available	680	580	600	180	460	500	620	810	430	430	5290
Eggs deposited:	348	356	242	52	228	290	301	166	199	271	2453
(*a*) In prepupae	215	244	178	39	182	223	242	100	128	178	1729
(*b*) In pupae	133	112	64	13	46	67	59	66	71	93	724
No. of hosts parasitized	243	194	127	32	104	166	166	104	125	138	1399

TABLE 3. OVIPOSITION OF *DIADROMUS COLLARIS* IN DAY-OLD *PLUTELLA* PUPAE

	Females										
	A	B	C	D	E	F	G	H	I	J	Totals
Length of life (days)	35	90	64	20	32	60	67	21	53	76	518
No. of hosts available	350	900	640	200	320	600	670	210	530	760	5180
No. of eggs deposited	134	190	339	137	127	395	298	104	407	236	2377
No. of hosts parasitized	66	146	184	80	102	196	194	62	228	168	1426

Although there was an apparent lack of ability to discriminate between parasitized and unparasitized hosts, the fact that females are able to select prepupae and pupae of different ages, suggested that the parasites might possess selective faculties in regard to hosts containing the more advanced immature parasite stages. Such behaviour has been noted with *Melittobia acasta* (Thompson and Parker 1927), *Cryptochaetum iceryae* (Thorpe 1930), and *Microplectron fuscipennis* (Ullyett 1936). Accordingly, the following experiment was set up. *Plutella* pupae containing late larvae and prepupae of *Diadromus collaris* were selected from the routine breeding cages and arranged in the normal manner. These were alternated with unparasitized pupae. Five pupae of each type were used for one female so that the arrangement was $\frac{PUPUP}{UPUPU}$, P representing a parasitized pupa and U an unparasitized pupa. This method of host disposition is the standard one adopted in "alternation" experiments. Ten females were each supplied with one set of hosts for 24 hr. at 20° C. in 4 × 1 in. glass tubes. After 24 hr. the pupae were dissected to determine the oviposition, and the results are given in table 4.

TABLE 4. THE REJECTION BY *DIADROMUS COLLARIS* OF PUPAE CONTAINING ADVANCED PARASITE STAGES OF THIS SPECIES

	Eggs laid in		
Exp. no.	Unparasitized pupae	Pupae containing advanced *D. collaris*	Total no. of eggs deposited
1	4 (4^1)	0	4
2	2 (2^1)	0	2
3	1	0	1
4	0	0	0
5	11 (1, 2, 4^2)	0	11
6	7 (1, 2^3)	0	7
7	16 (2^2, 3^1, 4^1, 5)	0	16
8	13 (2^2, 4^1, 5^1)	0	13
9	7 (1^3, 2^2)	0	7
10	7 (1, 2^3)	0	7
	68	0	68

The figures in brackets give the distribution of the parasite eggs in the five unparasitized pupae available at the commencement of the experimental period. Thus, in experiment 5, the figures 1, 2, 4^2, mean that in a total of four pupae parasitized, one contained one egg, one two eggs, and two each contained four eggs. When no figures are given other than the one number, all parasite eggs were laid singly. The precision with which the females avoided the hosts containing advanced parasite stages reinforced the suspicion that the superparasitism occurring in routine experiments was of a somewhat peculiar nature. Even in the present experiment, some females laid more than five eggs but failed to parasitize all five "normal" pupae available. Further, it appeared from an inspection of the figures giving the daily rates of oviposition of individual parasites, that the distribution of the eggs was worse than random, indicating that some factor or factors made certain hosts so attractive that they received a too generous quota of eggs.

Repetition of the foregoing discrimination experiment, using pupae containing second instar *Diadromus* larvae (which are approximately a quarter the size of the mature larvae), also gave a universal rejection of the parasitized material. However, it should be noted that in this experiment the age of the unparasitized pupae was 3 days younger than that of those containing the young parasite larvae, and although the development of a pupa is inhibited when a parasite is present, the disparity in the ages of the pupae available in the experiment may be partially responsible for the rejection of some hosts.

Finally, experiments were designed to test the possibility of the females being able to reject pupae containing eggs of *Diadromus*. The procedure consisted in supplying ten active parasites with three young pupae each spaced 1 in. apart in the usual container, and removing the females after a 3 hr. exposure had ensured that the majority of the pupae had been parasitized. Two fresh unparasitized pupae were then placed between parasitized pupae 1 and 2, and 2 and 3, making a total of five hosts. The whole set of five pupae were then left for 24 hr. so that the parasite eggs present would be well developed and could be separated from those which might be laid at any subsequent exposures to the females. This separation is easily accomplished because the freshly deposited egg is of a uniform opaqueness, while the day-old egg shows a differentiation including what appears to be the intestine of the developing embryo. Dissection must be done immediately after completion of the experiment.

On the expiration of the 24 hr., some of the cards with the five pupae were replaced in the same tubes as at the first exposure, while other cards were not placed in the tubes where they were at the first exposure. All cards were left for 1 hr. at 20° C with the females contained in the tubes. The placing with the same or different females at the second exposure was done with a view to noting whether the behaviour of the female differed in relation to hosts parasitized by herself or another female. The ovipositions at the second exposure are given in tables 5 and 6. Table 5 shows the layings when the same females were used at the first and second exposures in a series of ten experiments; table 6 gives the details where different females were used. There was, of course, no guarantee that all three hosts were parasitized at the first exposure, as there is no reliable means of determining this by external examination. Where such parasitization did not occur, more than two unparasitized pupae were available to the females at the second exposure, but this feature does not invalidate the conclusions to be drawn from the experiments.

TABLE 5. REJECTION BY *DIADROMUS COLLARIS* OF PUPAE CONTAINING DAY-OLD EGGS LAID BY THE SAME FEMALES

Hosts available to each female	Eggs laid in exp. no.										
	1	2	3	4	5	6	7	8	9	10	Totals
Parasitized pupae (3)	0	0	0	0	0	0	0	0	0	0	0
Unparasitized pupae (2)	2	4	2	3	2	2	5	4	3	1	28

TABLE 6. REJECTION BY *DIADROMUS COLLARIS* OF PUPAE CONTAINING DAY-OLD EGGS LAID BY DIFFERENT FEMALES

Hosts available to each female	Eggs laid in exp. no.										
	1	2	3	4	5	6	7	8	9	10	Totals
Parasitized pupae (3)	0	0	0	0	0	0	0	0	0	0	0
Unparasitized pupae (2)	1	3	4	3	2	5	3	3	2	3	29

Discrimination was pronounced and hosts containing parasite eggs 24 hr. old (at 20° C) were avoided by the females irrespective of the females which effected the parasitization. It will be observed that appreciable superparasitism occurred in the pupae which were unparasitized at the commencement of the experiment. It seems clear from these and previous results that the heavy superparasitism in this species occurs by the action of individual females upon encountering unparasitized hosts. Three hypotheses may be put forward to account for this disposition on the part of the *Diadromus* under the prevailing conditions. First, the female may be unable to distinguish between unparasitized hosts and those containing recently deposited eggs; secondly, she may lay more than one egg at any one act of oviposition; and, thirdly, she may have a tendency to remain in, or return to, the vicinity of a host which had recently been attacked by her. No method has yet been devised for testing the first hypothesis because of the difficulty of identifying eggs laid at the first and second exposures when the ovipositions occur within a short time of each other. It will be shown in experiments to be described later (see table 9) that with brief exposures of the host pupae to the females, the great majority of the hosts contained one egg, so that the second hypothesis is untenable and the first doubtful. This leaves the third suggestion as being the most likely.

As this behaviour might have resulted from confining the females in very small spaces, relatively large celluloid cages of 12 in. diameter and 5 in. height were constructed. These cages were capped with fine muslin. Ten pupae placed 2 in. apart in rows of five, were gummed on squared paper at the bottom of each cage. As controls ten pupae were placed at ½ in. intervals in cages 4 in. diameter and 5 in. height (in the routine 4 × 1 in. glass tubes, pupae are spaced ½ in. apart in two rows of five). A single female was placed in each cage for 24 hr. at 20° C, and a total of thirty-six such readings involving different females were taken for each type of cage. In the 4 in. diameter cages, 210 eggs were deposited in 132 hosts when 360 hosts had been available. In the 12 in. cages, 170 eggs were laid in 104 of the 360 hosts available throughout the experimental period. The degree of superparasitism is similar, and it is presumed that this disposition on the part of the females to lay more than one egg in hosts in the laboratory cannot easily be modified. A tendency to excessive superparasitism in the field has been described by Thompson and Parker (1930) with *Eulimneria crassifemur*, a larval parasite of the European corn-borer, and may be due to a similar intrinsic weakness.

The foregoing experiments have established the existence of a capacity by the female *Diadromus collaris* of avoiding ovipositing in hosts containing certain stages of the same species. This faculty may be conveniently termed *intraspecific* discrimination in contrast to *interspecific* discrimination, when the female parasite of one species is able to distinguish the hosts containing immature stages of a different species.

Prepupae encountered in the field by the Diadromid will frequently contain larval stages of the *Angitia* species, as these parasites emerge from the host after cocoon formation by the latter. As a preliminary experiment, prepupae which could be recognized by the observer as containing advanced larvae of *Angitia cerophaga*, were taken from the routine breeding cages and arranged in the standard manner in alternation with unparasitized prepupae. Ten females were each supplied with a choice of five of each host types for 24 hr. at 20° C. A total of seventy-five eggs was laid and all were placed in the unparasitized hosts. A similar result obtained when *Angitia cerophaga* cocoons within the *Plutella* cocoons were alternated with unparasitized prepupae. It was noted during the course of the experiments that such parasitized hosts were examined by the females.

The usual type of prepupa encountered by the *Diadromus* female is that containing an *Angitia* larva at least half-grown. The majority of the host larvae are attacked in the early instars by the *Angitia* females so that at the time of cocoon formation by the host the contained parasite is in the second or third instar. However, in order to test the interspecific discriminatory faculties of the *Diadromus* thoroughly, it was decided to supply the females with prepupae containing first instar larvae of *Angitia cerophaga*. They will, of course, sometimes meet such conditions in the field. Hosts containing such young stages of the *Angitia* were obtained in the following manner. Ten fully-grown larvae of *Plutella* were confined in 4 × 1 in. tubes containing a strip of cabbage. A single female *Angitia cerophaga*, which had not previously oviposited, was left with them for 6 hr. at 20° C. The mature larvae soon spin their cocoons, and the prepupae which are unparasitized transform into pupae within 24–36 hr. at the prevailing temperature. The prepupae which remain as prepupae longer than this period are usually parasitized, the presence of the parasite stage or some action of the ovipositing *Angitia* preventing pupation. This inhibition of pupation is not invariable, as occasionally pupae containing *Angitia* eggs have been obtained. It is not known if any development takes place in this medium, but it is unlikely in view of the time which must have elapsed since oviposition. Incidentally, this action of *A. cerophaga* in preventing pupation contrasts with that of *Diadromus collaris*, which oviposits readily in the prepupae, which in turn is followed after the normal interval by the pupal instar.

These parasitized prepupae were left for 4 days at 20° C to allow development of the parasites to primary larvae and were then alternated in the usual way with unparasitized prepupae in 4 × 1 in. tubes. Ten such experiments were performed. The eggs laid by the females are given in table 7.

Of the fifty hosts which were thought to have been parasitized by *Angitia cerophaga*, some fifteen did not contain parasite larvae. Eleven eggs were laid by the *Diadromus* females in some of these latter unparasitized prepupae, but these figures have not been included in the above table. It will be observed that occasionally the females do oviposit in prepupae containing *Angitia* stages, and such multiparasitism may occur even when unparasitized hosts are also available.

Confirmation of the tendency to avoid ovipositing in prepupae containing stages of another species of parasite was obtained by confining females with such hosts and providing no choice. In some respects this arrangement gives a better approximation to field conditions, where the females are rarely confronted with a choice of hosts at very close range. Prepupae parasitized by *A. cerophaga* were obtained by the usual routine, and the parasite larvae were allowed to develop to the second instar. Ten females were each supplied with five such hosts for 2 hr. at 20° C. At the end of this period the females were given five unparasitized prepupae for 2 hr., then parasitized prepupae again for 2 hr., and finally unparasitized prepupae for the fourth 2 hr. period. All the *Diadromus* females used in this experiment had been observed actively ovipositing but had not laid for 2 days previous to the experiment. Thė oviposition of the females is shown in table 8. All

TABLE 7. DISCRIMINATION BY *DIADROMUS COLLARIS* WHEN GIVEN A CHOICE OF UNPARASITIZED PREPUPAE AND PREPUPAE CONTAINING YOUNG *ANGITIA CEROPHAGA* LARVAE

	Eggs laid in		
Exp. no.	Unparasitized prepupae	Prepupae containing young *cerophaga* larvae	Total no. of eggs laid
1	9 (3^3)	0	9
2	7 (1^2, 2^1, 3^1)	2 (2^1)	9
3	8 (1, 2^1, 5^1)	3 (1, 2^1)	11
4	5 (1, 2^2)	0	5
5	7 (1^2, 5^1)	0	7
6	11 (2^3, 5^1)	0	11
7	9 (1^3, 3^2)	1	10
8	8 (1^2, 2^1, 4^1)	0	8
9	10 (1, 2^1, 3^1, 4^1)	0	10
10	10 (2^2, 3^2)	0	10
	84	6	90

TABLE 8. OVIPOSITION OF *DIADROMUS COLLARIS* IN PREPUPAE CONTAINING SECOND INSTAR LARVAE OF *ANGITIA CEROPHAGA*

	Eggs laid in			
Exp. no.	Exposure 1 Parasitized prepupae	Exposure 2 Unparasitized prepupae	Exposure 3 Parasitized prepupae	Exposure 4 Unparasitized prepupae
1	0	6 (1^2, 2^2)	0	4 (1^2, 2^1)
2	0	3	0	4 (1^2, 2^1)
3	0	5 (1^2, 3^1)	0	4
4	0	5 (1, 2^2)	0	4 (1^2, 2^1)
5	0	4	0	2
6	0	4 (1^2, 2^1)	0	5 (1, 4^1)
7	0	6 (1^2, 2^2)	0	2 (2^1)
8	1	6 (1^3, 3^1)	0	5 (1, 2^2)
9	0	2	0	5 (1, 2^2)
10	0	10 (1^2, 2^1, 3^2)	0	2

unparasitized prepupae were examined under the binoculars before dissection, and all appeared to the observer to be equally accessible by the terminal holes of the silken cocoons.

All but two of the parasitized prepupae actually contained *Angitia* larvae, but for some reason these unparasitized pupae were not oviposited in by the *Diadromus*. The exercise of restraint is evident. During the course of the experiments all females were observed to examine the parasitized prepupae, insert the ovipositor into the host via the terminal hole of the cocoon, and then quickly withdraw the ovipositor again. The duration of this action was much more brief than that of the usual act of oviposition. After a few such attempts, the females became very agitated and moved rapidly round the tubes, and frequently ran over the hosts in their movements. When, later in the experiment, contact was made with some host in the supply of unparasitized prepupae, the parasites stopped and examined them and eventually oviposited. It appears, therefore, that these females are able to determine the nature of their hosts partly by external examination (probably with the antennae) and partly by internal examination with the ovipositor.

For purposes of comparison a parallel series of experiments on the exercise of restraint was done with pupae containing second instar larvae of *Diadromus collaris*. These were obtained by exposing sets of two pupae spaced 1 in. apart for 2 hr. with females and leaving the hosts at 20° C for 3 days. Owing to the peculiar oviposition habits of this parasite it was decided to limit the number of hosts exposed to the females so as to be tolerably certain of securing parasitism of all hosts. A heavy superparasitism is inevitable, but usually one parasite larva soon assumes ascendancy. It was further decided to restrict the exposure of such parasitized hosts to as short a time as would permit of examination and oviposition by the females. A period of 20 min. was selected, and immediately on expiration of this the females were given sets of two unparasitized pupae. This process was repeated ten times with ten females. The numerous changes of the host cards did not seem to disturb the parasites to any appreciable extent. The behaviour is summarized in table 9. Exposures 1, 3, 5, 7, 9 were to parasitized pupae and those of 2, 4, 6, 8, 10 to unparasitized pupae. The experiment thus involved a period of 3 hr. 20 min. The females had not been supplied with hosts for 3 days previous to the experiment.

The dashes in the Table indicate that no eggs were laid. It is a remarkable fact that in this experiment and all preceding not a single case of superparasitism by *Diadromus* females has occurred, provided the host parasitized contained a *Diadromus* stage more than 24 hr. old. The restraint of the female in respect of hosts parasitized by *Diadromus* seems to be more rigid than when the hosts are prepupae containing *Angitia cerophaga* larvae. The extent to which the female refrains from laying in parasitized hosts under field conditions is unknown and difficult to determine, but the results of dissections of some field collections of pupae are discussed later in this paper.

TABLE 9. OVIPOSITION OF *DIADROMUS COLLARIS* IN PUPAE CONTAINING SECOND INSTAR LARVAE OF THIS SPECIES

Exp. no.	Eggs laid at exposure no.										Eggs laid in		
	1	2	3	4	5	6	7	8	9	10	Unparasitized hosts	Parasitized hosts	Total laid
1	—	2 (2^1)	—	1	—	4 (1, 3^1)	—	1	—	2	10	0	10
2	—	1	—	0	—	0	—	0	—	0	1	0	1
3	—	2	—	0	—	1	—	0	—	2	5	0	5
4	—	1	—	1	—	0	—	2	—	0	4	0	4
5	—	1	—	1	—	0	—	1	—	1	4	0	4
6	—	1	—	2	—	1	—	1	—	1	6	0	6
7	—	0	—	0	—	1	—	0	—	1	2	0	2
8	—	3 (1, 2^1)	—	1	—	2 (2^1)	—	1	—	2 (2^1)	9	0	9
9	—	2	—	2	—	1	—	0	—	1	6	0	6
10	—	1	—	1	—	2	—	1	—	2	7	0	7
											54	0	54

It has been suggested by the present author (1938) that, with the Chalcid *Ooencyrtus kuvanae*, a decrease in the facilities for oviposition (such as might obtain from scarcity of hosts, inclement weather for the activity of the female, etc.) may result in greater oviposition activity at a later time in life when conditions become favourable. An attempt was made to indicate the importance of this feature by preventing females from laying for various periods and then noting any change in behaviour when confronted with parasitized material. It was shown that when no hosts were available over several days, mature eggs accumulated in the oviducts and these were eventually released with little consideration for the nature of the hosts available. Normally this parasite has good discriminatory faculties and limits its laying in the presence of parasitized hosts.

This characteristic of alteration of behaviour in consequence of the condition of the gonads does not apply to *Diadromus collaris*. In the foregoing experiments to test the exercise of restraint, all the females had been prevented from ovipositing for at least two days. The numbers of eggs eventually laid by these females were not appreciably greater than those obtained in the usual daily oviposition in unparasitized material. Confirmatory figures have been had from the other experiments.

It has been noticed that the female Diadromids feed on the host contents at irregular intervals throughout their lives (figure 3, plate 18). This feeding habit is quite pronounced and is particularly evident if the parasites have been kept from hosts for some time. The female punctures the pupa with her ovipositor at the head or tail end, having first enlarged the terminal holes in the cocoon by tearing with the mandibles. The latter operation is necessary to permit of the insertion of the female's head between the cocoon and the surface of the host. Such behaviour has not been observed prior to normal oviposition. Sometimes, a female enters the cocoon and punctures the host (figure 6, plate 19). Frequently pupae are so mutilated by this treatment that no emergence occurs. Unfortunately, any advantage which might accrue from this predatory habit of the adult is partially nullified by the fact that the feeding is indiscriminate, parasitized hosts being as acceptable as unparasitized.

The daily rate of oviposition of the *Diadromus* females, at 20° C for the first 14 days of life, is shown in figure 7. This rate was obtained by averaging the oviposition of twenty females. Maximum activity is reached about 1 week after emergence. Each female was supplied with ten hosts daily, and was therefore able to feed on host contents at any time during the first 14 days of life. It was found that with a set of ten females, which immediately on emergence had been kept without hosts for 14 days and then supplied daily with unparasitized hosts, the oviposition curve was of a similar character to that in the first instance (figure 7). Further sets of females kept without hosts for 6 or 28 days after emergence also gave layings which increased gradually in the manner of figure 7. Thus twelve females kept without hosts for 6 days after emergence laid twenty-three eggs in the first day's supply of hosts, forty-three in the second and third days' supply, and seventy-two eggs in the fourth day's exposure. Prior to access to pupae all these females had been confined in glass cages of 4 in. diameter and 5 in. height and fed on moist sugar and a split raisin. When they were eventually given pupae, they naturally fed, at intervals, on the contents. It would appear from these figures that the feeding of the adult on the host contents is associated with the normal development of the gonads. This apparent need for protein was verified by dissections of the gonads of freshly emerged females and those which have been fed for 6 or 14 days on a diet of sugar and raisins and given no hosts. In all cases the ovarioles were usually well developed but very few mature eggs were present.

This feature helps to explain the absence of accumulation of mature eggs in the oviducts, which was inferred from the experiments on restraint. It has been noted above that the females in these experiments had been prevented from ovipositing for several days before use, but did not lay more eggs than those obtained in routine daily ovipositions. From this viewpoint it is therefore very doubtful whether, in this species, irregularity in the availability of hosts in the field will effect the incidence of superparasitism and multiparasitism.

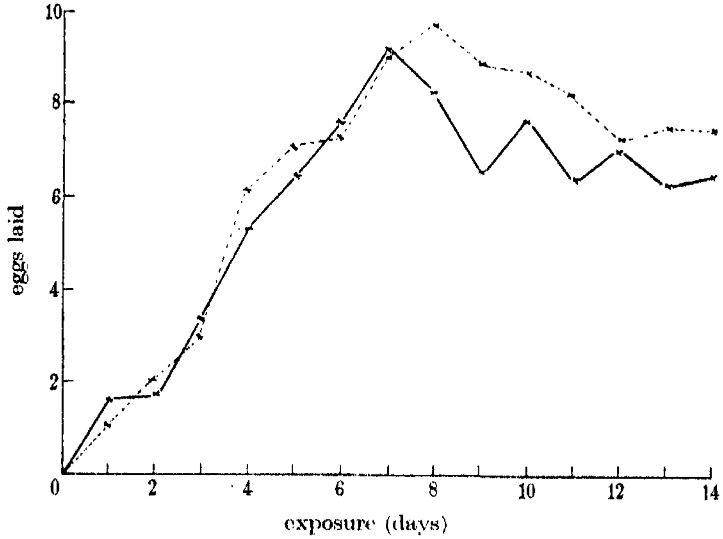

FIGURE 7. The effect of pupal feeding on oviposition by *Diadromus collaris*. The barred curve gives the average daily oviposition when the females were supplied with pupae immediately on emergence. The dotted curve gives the average daily oviposition when the females had been kept for the first 14 days of life without pupae.

At this stage it will be advantageous to summarize briefly some of the factors which have been found to influence the oviposition response of *Diadromus collaris*. These factors may be broadly classified as (1), those involving the silken cocoon surrounding the host, and (2), those involving the physiological condition of the host. Although the silken cocoon is of significance because of the nature of the oviposition act, the behaviour with "naked" hosts is probably of little practical importance, since only a very small percentage of field pupae are not covered by cocoons. Moreover, as was concluded by Thompson and Parker (1927) with *Melittobia acasta*, the oviposition behaviour of the female parasite does not depend so much upon the character of the envelope surrounding the hosts as on what lies beneath it. Unparasitized hosts are more acceptable when histogenesis is in the early stages, but hosts parasitized in such conditions are usually rejected by subsequent females. This rejection is very well defined in respect of pupae parasitized by *Diadromus collaris* itself, and prepupae containing the advanced larval stages of *Angitia cerophaga*. Superparasitism in prepupae and pupae seems to occur only in cases where the host contains recently deposited eggs; multiparasitism obtains when the *Diadromus* female encounters prepupae containing very early instars of *Angitia cerophaga*, but oviposition in these conditions is restricted. Experiments suggest that feeding on the host contents is necessary for the full development of the gonads.

(*b*) *Angitia cerophaga* Gravenhorst

This species of *Angitia* is a very common larval parasite of *Plutella maculipennis* and has a world-wide distribution. The female is very active but seems to have no precise sense guiding her to the host larvae; her search of a leaf with host larvae consists mainly of an incessant tapping of the leaf surface with the antennae and tip of the ovipositor. Immediately a *Plutella* larva is touched it moves, and the parasite gives a quick jab and an egg is laid. Sometimes the host larva is impaled on the ovipositor and not easily removed. When a larva falls to the bottom of a breeding jar, the female parasite often drops after it and parasitizes it, but it has been noticed that a female is unable to locate a stationary host caterpillar on the bottom of the container, or on a leaf. However, this latter contingency is infrequent as most *Plutella* larvae are very sensitive and react quickly to movements in their vicinity.

The female *Angitia cerophaga* attacks all stages of the host larva with equal facility when confined with them in the routine 4 × 1 in. glass tubes. A strip of cabbage is so wedged in the tube that the hosts are unable to escape contact with the parasite by lying between the cabbage and the sides of the tube. Selected larval stages are then placed in the tube and left for a few hours to resume normal feeding before the parasite is introduced (figure 3, plate 18). Table 10 gives the results of exposing five second-instar larvae and five mature (fourth-instar) larvae to *Angitia* females for 6 hr. at 20° C. Some of the final-instar larvae had spun cocoons by the conclusion of the experiment. It has been observed that the females also oviposit occasionally in prepupae enclosed within cocoons. This reaction depends upon movement by the prepupa when the parasite walks over the cocoon; if there is no movement, the female takes no interest in the contents.

The ready acceptance of final-instar larvae immediately prior to their spinning up and even when in cocoons, is surprising in view of the very heavy mortality of the parasites in such cases (see section dealing with mortality in superparasitism). Hundreds of mature *Plutella* larvae have been dissected in the field, and these rarely contained stages younger than second-instar larvae of *Angitia*; there is little doubt that normally the advanced host larvae escape parasitization by their extreme agility, but some may be attached during cocoon formation. Tapping a leaf surface causes the larger hosts to wriggle quickly off the leaf and suspend themselves by a silken thread. As has been stated, similar behaviour occurs in the laboratory rearing cages, but in a small tube this agility is largely nullified. The younger larval stages of *Plutella* are usually loosely enclosed by silken threads on the leaf surface and are more restricted in their movements.

TABLE 10. THE HOST INSTARS ATTACKED BY *ANGITIA CEROPHAGA*

Instars available to each female	Eggs laid in exp. no.										Total no. of eggs laid
	1	2	3	4	5	6	7	8	9	10	
2nd	9	7	7	5	8	4	4	8	3	3	58
4th	16	6	10	5	2	3	1	6	4	1	54

To ascertain the intraspecific discriminatory faculties, if any, of this species, a series of ten young second-instar *Plutella* larvae were placed in ten tubes and a single female *Angitia* was confined in each tube for 5 hr. at 20° C. These females had not oviposited previous to the experimental period. Hosts so treated were then left for 48 hr. at 20° C, so that the contained parasite eggs were on the point of hatching. The majority of the hosts were still in the second instar and were then again exposed to a fresh set of *Angitia* females from the same source as the first for 1 hr. at 20° C. This second exposure was undertaken to obtain some indication as to whether the species restricted oviposition in the presence of parasitized host material. As the adult parasites were from the same source with only a slight age difference of 2 days, the eggs deposited at the first exposure may be regarded as a control for those laid at the second exposure. Separation of these egg depositions is easy because by the time dissection is performed the first laid eggs have hatched. Records of the eggs found in each host are given in table 11. Large bars (/) indicate the first ovipositions and small bars (′) the second ovipositions.

Inspection of these details established (1) that the *A. cerophaga* female is able to detect hosts parasitized by either herself or another female of the same species and can avoid ovipositing in them; and (2) that the females limit their laying when the majority of the hosts available are parasitized. This selective faculty is rather remarkable because, when disturbed by the actions of the female parasite, the host larvae keep moving in the tubes and hence the position of any unparasitized hosts relative to those already parasitized is constantly changing throughout the course of such an experiment. This feature is well brought out in those cases in the experiment where the first females parasitized eight or nine hosts and the subsequent females attacked the few unparasitized hosts available in addition to laying in the parasitized material. The presence of these few unparasitized hosts complicated, to a slight extent, the examination of restraint, and the total number of eggs laid in the parasitized hosts should be taken as slightly less than that indicated in the table. In connexion with this possession of discriminatory faculties, it is of interest to note that Chamberlin and Tenhet (1926) state that there appears to be a marked tendency on the part of the Microgasterine Braconid, *Cardiochiles nigriceps* Viereck, to avoid laying in second-instar larvae of the tobacco bud worm, *Heliothis virescens* Fabricius, which have previously been parasitized. Frequently a female was observed to return to a tobacco plant which she had left a short time previously, but she ignored the larvae which she had parasitized on her first visit. Moreover, females often refused to oviposit in the first-instar larvae, which is the "preferred" size, and it was inferred that these hosts had been parasitized. These observations are particularly interesting in view of the fact that they were made in the field.

TABLE 11. OVIPOSITION BY *ANGITIA CEROPHAGA* IN HOSTS CONTAINING EGGS OF THIS SPECIES

Exp.	Eggs laid in host no.										Eggs deposited	
no.	1	2	3	4	5	6	7	8	9	10	1st laying	2nd laying
1	/	/ı	/	/	/	ıı	/	/	/	/ı	9	4
2	/	//	/	/	//ı	/	/	/ıı	/	/	12	3
3	//ı	//	/	//	////	/	//	/ııı	/	///	19	3
4	/	/ıı	/ıı	/	ıı	ı	/	/ı	/ı	/ı	8	10,
5	//ı	/ı	/	ı	/ı	/	/	/	//	?	10	4
6	/	/ı	/ı	/ı	/ıı	/ı	/ı	/ıı	/	/ı	10	10
7	//	//ı	/	/ı	/ı	/	/	/ı	/ı	/	12	5
8	/	/	//ı	//	//	/	/	/ı	///ıı	ı	14	5
9	ı	/ıı	/	/	//ıı	/ı	/ı	/	/	/	10	7
10	/	/	/	/	/	/	/	/	/	/	10	0
											114	51

Having established the existence of the faculty of partially refraining from ovipositing in young host larvae containing *Angitia cerophaga* eggs, experiments were next undertaken to ascertain the effect of larger hosts containing more advanced parasite stages on oviposition. The number of second-instar larvae available in the tubes was reduced to five so as to obtain maximum parasitism of the hosts at the first exposure. Such larvae were then left for 4 or 6 days at 20° C, so that the contained parasites developed to the first and second instars. During this interval the hosts had become third instars (containing first instars of *cerophaga*) and fourth instars (containing second instars of *cerophaga*). Controls, consisting of unparasitized third and fourth instars of *Plutella* of sizes corresponding to those containing the parasite larvae, were obtained from the routine breeding cages. Other procedure was as in preceding experiments. The *Angitia* females used had not previously oviposited and were confined in glass tubes 4 × 1 in. for 1 hr. at 20° C. Figures for the oviposition in hosts containing primary larvae of the *Angitia* are given in table 12, and those in hosts containing second-instar larvae of the *Angitia* in table 13. The ovipositions in the control tubes have also been incorporated in these tables. Egg laying at the first and second exposures is indicated by large and small bars respectively.

TABLE 12. OVIPOSITION OF *ANGITIA CEROPHAGA* IN HOSTS CONTAINING FIRST-INSTAR LARVAE OF THIS SPECIES

Exp. no.	Eggs laid in parasitized host no.					Total eggs laid in	
	1	2	3	4	5	Parasitized hosts	Control (unparasitized)
1	/ı	/	/ı	/	/	2	13
2	/	/ı	/ıı	/ıı	/ıı	7	8
3	/ıı	/ı	/ı	/	/	4	8
4	/ı	/ı	/	/	/ı	3	4
5	///ı	/	//	/ı	/	2	3
6	/ı	/	/ıı	//	/	3	11
7	/	/	/	/ı	/	1	4
8	/	/	/	/	/	0	8
9	//	/	/ı	/	/	1	6
10	/	/ı	/	/	/	1	7
						24	72

It will be seen that in the experiments designed to test restraint, all hosts with the exception of the two indicated in table 13, were parasitized at the first exposure. The females restricted their laying and this limitation may be more pronounced when the hosts contain more advanced parasite stages.

However, for reasons already stated, the later instars of the host larva usually escape attack in the field so that this rejection is largely of academic interest. It might be mentioned that the ovipositor appears to be the most important organ involved in the determination of the nature of the host. Hosts containing larvae of *Angitia* will almost invariably have moulted since the time of attack by the female parasite, so that there is no question of some material traces of the original female being left on the surface of the host and detected by the subsequent female as was demonstrated by Salt (1937) with *Trichogramma evanescens* and its host *Sitotroga cerealella*.

TABLE 13. OVIPOSITION OF *ANGITIA CEROPHAGA* IN HOSTS CONTAINING SECOND-INSTAR LARVAE OF THIS SPECIES

Exp. no.	Eggs laid in parasitized host no.					Total eggs laid in	
	1	2	3	4	5	Parasitized hosts	Control (unparasitized)
1	/	/ı	/	/	/	1	0
2	//	/ı	/	/	/	1	8
3	ı	/	/	/	/	1 (−1)	9
4	/ı	/	/	/	/	1	9
5	/	/	/	/	/	0	8
6	/ı	/ıı	/	/	/	3	1
7	/ı	/ı	/ı	/	/	3	7
8	/	/	/	/	/	0	10
9	//ı	/	ı	//	/ı	3 (−1)	9
10	/	//	//ı	/ı	/	2	11
						15 (−2)	72

Experiments to determine the interspecific discrimination of *Angitia cerophaga* females when some of the hosts available contain immature stages of *Apanteles plutellae* have been hampered by the erratic oviposition of the *Apanteles* in the laboratory. Even when the species did lay during the course of an experiment, the numbers of eggs laid were frequently too small to provide a good test of the selective faculties of the *Angitia* females. The figures for the *Apanteles* layings given in table 14 are the best obtained from a total of thirty investigations. The procedure was as follows: Ten second-instar *Plutella* larvae were confined in 4 × 1 in. tubes with *Apanteles plutellae* females for 3 hr. The parasites were then removed and fresh *Angitia cerophaga* females substituted and left for 3 hr. at 20° C. Discrimination was therefore tested when the hosts contained freshly deposited eggs—perhaps the most difficult conditions for the operation of this faculty.

It will be seen that the results have been arranged in terms of the numbers of hosts parasitized at the first and second exposures. As there was very little superparasitism and multiparasitism the majority of these figures also represents the actual ovipositions. The exceptions are shown by the figures in brackets, which give the number of eggs laid by a female in parasitizing the indicated number of hosts. The combined activity of the *Angitia* and *Apanteles* females gave a varying parasitism and in only one experiment were all ten hosts utilized. Although the ovipositions by either species were small, the data suggest a good selective faculty in respect of hosts parasitized by *Apanteles*. Owing to the difficulty of obtaining good oviposition by the *Apanteles* no experiments have been done with hosts containing larvae of this species. However, since the *Angitia* female is able to reject hosts containing larvae of its own species, it may be presumed that the interspecific discriminatory faculties of *Angitia* also operate throughout the range of the *Apanteles* development in the host.

TABLE 14. DISCRIMINATION BY *ANGITIA CEROPHAGA* WHEN SOME OF THE AVAILABLE HOSTS CONTAIN EGGS OF *APANTELES PLUTELLAE*

Exp. no.	No. of hosts parasitized by *Apanteles*	Unparasitized hosts available	No. of parasitized hosts used by *Angitia*	No. of unparasitized hosts used by *Angitia*	Total hosts parasitized
1	2	8	0	2	4
2	1	9	1	6	7
3	4	6	0	2	6
4	6	4	0	2	8
5	2	8	0	5	7
6	6	4	5	4 (5)	10
7	3 (4)	7	0	2	5
8	3	7	0	4	7
9	4 (5)	6	0	3	7
10	3	7	0	5	8

The outstanding feature of the oviposition response of *Angitia cerophaga* is the apparently wide range of factors to which reaction is made. It is unusual for a hymenopterous larval parasite to oviposit in all instars of the host larvae. The physiological host conditions involved include such diverse types as that of the unparasitized first instar, the prepupa in a state of histolysis, the instars containing immature parasites of *Angitia*, and instars parasitized by *Apanteles plutellae*.

(c) *Apanteles plutellae* Kurdjumov

This small Braconid has a geographical distribution in Europe which compares favourably with that of the *Angitias* and *Diadromids* but has only been found to parasitize a very small percentage of the larval population of *Plutella*. It is a curious fact that this smallness of the *Apanteles* population relative to that of the *Angitia* spp., has been paralleled in laboratory breeding. It has not yet been possible to get the *Apanteles* to breed freely under laboratory conditions; it lays rather sporadically and gives rise to few progeny. In view of these oviposition habits, experiments with this species have been somewhat unsatisfactory.

The methods of searching for, and attack of, host larvae are very similar to those of *Angitia cerophaga*. The movements of the host again play an important part in the localization of the latter by the female. The females readily attack and oviposit in the first three larval instars of the host and although they attempt to attack the final instar, the violent reactions of the mature host larva prevent any ovipositions as the small parasite is quickly thrown off. No eggs have been found in such larvae after confinement with the parasites. As the mature *Apanteles* larva emerges from the fourth instar of *Plutella* to spin its cocoon this failure is not surprising. Experiments with *Apanteles* females in the routine breeding cages have shown that the first and second host instars are the most suitable for oviposition and subsequent development of the parasite.

Tests of interspecific discrimination were made by supplying the females with ten early second-instar hosts, each in the usual containers, for 3 hr. at 20° C. The figures in table 15 are the highest ovipositions obtained from several series.

TABLE 15. OVIPOSITION OF *APANTELES PLUTELLAE* IN SECOND-INSTAR *PLUTELLA* LARVAE

	Experiment no.										
	1	2	3	4	5	6	7	8	9	10	Totals
No. of eggs laid	2	5	6	7	5	6	8	3	5	4	51
No. of hosts parasitized	2	5	6	5	5	6	8	3	4	3	47

Selection here appears to be on a par with that of *Angitia cerophaga*. Difficulty in securing a good supply of hosts containing larvae of *Apanteles* has prevented an extension of this experiment.

Behaviour, where some of the available hosts are parasitized by *Angitia cerophaga*, was examined by the procedure outlined for interspecific discrimination in the *Angitia*. The sets of ten hosts contained recently deposited eggs of *A. cerophaga* and the *Apanteles* females were left in the tubes for 3 hr. at 20° C. The difficulty in this experiment was to limit the laying of the *Angitia* females so that a fair number of unparasitized hosts was present for the *Apanteles*. The best series obtained was one in which six or more of the ten hosts had been parasitized by the *Angitia* at the first exposure. This series is comparable with that given in table 14, but it should be noted that in the latter case the parasitism at the first exposure was not very heavy. The *Apanteles* females were therefore confronted with more difficult conditions. The behaviour of the *Apanteles* in such conditions is shown in table 16. Except where indicated by figures in brackets, the numbers of hosts attacked also indicate the numbers of eggs deposited.

TABLE 16. DISCRIMINATION BY *APANTELES PLUTELLAE* WHEN SOME OF THE AVAILABLE HOSTS CONTAIN EGGS OF *ANGITIA CEROPHAGA*

Exp. no.	Hosts used by *Angitia*	Unparasitized hosts available	No. of parasitized hosts used by *Apanteles*	No. of unparasitized hosts used by *Apanteles*	Preference ratio for unparasitized hosts
1	6 (8)	4	1	0	—
2	7	3	1	0	—
3	7 (12)	3	0	1 (2)	—
4	9 (11)	1	1	0	0·88
5	7	3	1	1	—
6	7 (10)	3	2	0	—
7	9	1	1	1	1·64
8	1	9	0	4 (6)	—
9	7	3	2	2	2·00
10	8 (9)	2	3	2	2·67
11	6 (9)	4	3 (4)	3	—
12	8	2	5	1	1·17

Taking the average of all the experiments the *Apanteles* show a preference for unparasitized hosts in the ratio of 1·6 : 1. The preference ratio for the first four experiments of hosts parasitized by *Angitia* may be due to chance or to a definite rejection by the four females. The latter reason is unlikely. It is apparent, then, that the *Apanteles* females will readily lay in hosts parasitized by *Angitia cerophaga*. Thus, when no unparasitized hosts are available they oviposit in hosts containing larvae of the *Angitia*. Details are given in table 17 of an experiment in which ten females were each supplied with five third-instar *Plutella* larvae containing first-instar *Angitia* larvae for 2 hr. at 20° C. Control females were each given five unparasitized third instars.

It is concluded from these few experiments that the oviposition response of *Apanteles plutellae* is of a similar nature to that of *Angitia cerophaga*, but the range of host instars attacked is more restricted. It is possible also that this species is somewhat more tolerant of the physiological conditions of the instars so that the females oviposit more readily in parasitized hosts.

TABLE 17. OVIPOSITION OF *APANTELES PLUTELLAE* IN HOSTS CONTAINING FIRST-INSTAR LARVAE OF *ANGITIA CEROPHAGA*

	Eggs laid in experiment no.										
Hosts available	1	2	3	4	5	6	7	8	9	10	Totals
Unparasitized larvae (control)	0	1	3	5	1	2	1	3	0	2	18
Parasitized larvae	5	0	0	0	3	2	5	0	0	2	17

No attempts have yet been made to determine the effects of delaying facilities for oviposition on the incidence of multiparasitism in the case of the species of *Angitia* and *Apanteles*. It is intended to examine this feature in a later paper. In all foregoing experiments the females which were used had not oviposited previous to the experimental period. Some of the *Angitia cerophaga* females were left 14–21 days before use, but the discriminatory faculties showed no appreciable difference from the younger females. Superparasitism was frequent as often too few hosts were available for the parasites.

4. MORTALITY IN SUPERPARASITISM AND MULTIPARASITISM

Only one adult emerges from the host in the case of *Angitia cerophaga*, *Apanteles plutellae* and *Diadromus collaris*. When more than one specimen of either *Apanteles* or *Angitia* occurs in a host (that is, superparasitism) one larva only survives to the second instar. In the foregoing experiments, the supernumerary eggs had been deposited within a period of 3 or 4 hr., and, in practically 100% of the cases examined, one living first instar was present and the other inhabitants were in various stages of degeneration. Hence, elimination of rivals in both these larval parasites occurred in the first instars.

Forty-four dissections of hosts which contained one *Apanteles* and one *Angitia* larva gave only seven instances in which both parasites were alive. These hosts had been parasitized in the second instar and were dissected in the late third and early fourth instars, so that the contained parasites had developed to the late first instar. In the other thirty-seven hosts, seventeen contained a dead first instar of *Apanteles* and twenty dead primary larvae of *Angitia*. This indicates that when the two species start development at the same time in a host, neither parasite has a marked intrinsic superiority. Data available at present are insufficient for conclusions on mortality when the ovipositions by these species occur at an interval of more than 24 hr. The death of supernumerary primary larvae appears to be due to physiological causes so that the survival of the older inhabitant is probable. It must be remembered that the nature of the selective and restraint faculties possessed by the *Angitia* and *Apanteles* females tends to concentrate superparasitism and multiparasitism in the early stages of parasite development in the host and therefore cases where the two species within one host are likely to be of widely separated ages will presumably be infrequent. Further, development of the early stages of the parasite is slow and accompanied by a fair increase in size of the hosts, which for reasons previously stated, are then not so easily attacked by the adult parasites.

If two or more specimens of *Diadromus collaris* occur in a pupa, the supernumerary larvae die in the first or second instars. Where eggs or young larvae of *Angitia cerophaga* and *Diadromus collaris* are present in the same prepupa neither species survives. The *Diadromus* is unable to develop in prepupae while it is extremely rare for *Angitia cerophaga* to pupate successfully if the egg has been laid in the later stages of the final instar of the larva, or in the prepupae. At 20° C, the *Angitia* larva emerges from the prepupa 3 or 4 days after cocoon formation by the host. If the parasite stage is so young at the time of spinning up of the host that more than a week would elapse before the larva could mature, the host succumbs before parasite development can be completed. Prepupae containing late larval instars of *A. cerophaga* are very rarely attacked by *Diadromus*.

The question of the method of elimination of rival larvae by the successful parasite in a given host has received attention from numerous workers. It has been shown that this destruction may be brought about by actual combat (Pemberton and Willard 1918), physiological inhibition (Spencer 1926), or starvation (Fiske 1910). In the three parasites studied in the present investigation actual combat and physiological inhibition have been involved. *D. collaris* has three larval instars and larvae in the first and second instars have frequently been found impaled in the sickle-like mandibles of another larva of this species. It is probable that this is the usual means of suppression in this form, although undeveloped eggs and dead larvae with no trace of mechanical injury are sometimes found. Occasionally a single egg or larva is found in a melanized condition in a pupa, and this may perhaps be attributed to host reaction. In the case of

Angitia cerophaga and *Apanteles plutellae*, the factors appear to be of a biochemical nature as no instance of injury or actual combat has been observed. When several larvae of one species occur in the same host, only one first instar remains alive, while the others are dead and later melanized. This condition obtains in superparasitism with either species and also in multiparasitism of the two forms. It has been noted that if *Apanteles* eggs are laid in hosts containing primary larvae of the *Angitia*, these latter are still alive and active 24 hr. later. The possibility that the death of the young parasite stages is due to the injection of poison by any subsequent ovipositing female is thus unlikely. In any case, such a cause could not operate in cases of superparasitism and the similar results obtaining in such conditions suggest that the factors responsible for the destruction of supernumerary primary larvae may be to some extent independent of the nature of these larvae.

5. Superparasitism and multiparasitism in field samples

Collection of data on field parasitism in North Holland in the summer of 1939 was interrupted by the outbreak of war, and it is thought advisable to include the few figures available to date in the present paper. Observations were made in the district of Lutjebroek, North Holland, where previous laboratory work had indicated the presence of all important primary parasites of this pest. Throughout the summer and autumn this part of Holland (De Streek) is given over almost exclusively to cauliflower cultivation and it has been found that the area is a very favourable one for work with *Plutella maculipennis*. The moth occurs here in fair numbers every year, whereas in England its incidence seems more sporadic.

The following host samples (tables 18 and 19) were obtained by random collecting from cabbage seed-beds, which are left standing until the late summer. These beds sometimes cover 40 or 50 square yards. The collections were made from plots more than a quarter of a mile apart, and approximately the same amount of time was taken for each. The figures for the *Angitia* spp. are grouped together because no means of separating the eggs and larvae of *cerophaga* and *fenestralis* has yet been found; emergence from collections made at the same time as the foregoing showed *cerophaga* to be much the more common species. It is very probable that no *Diadromus subtilicornis* was present in the beds examined as only *D. collaris* emerged from samples of pupae collected at the same time as the pupae for dissection. At the time of collection of these samples practically no emergence from

Plutella pupae, *Angitia* cocoons, etc., was observed and the figures are therefore those for the first generation of the host on cultivated crucifers.

Table 18. Field parasitism of mature first-generation larvae of *Plutella maculipennis* in North Holland, 1939

Sample no.	No. of larvae dissected	Larvae parasitized
1. D. Lighthart	114	46 each with 1 specimen of *Angitia* spp. 2 each with 1 specimen of *Apanteles plutellae*
2. J. Slaghter	100	41 each with 1 specimen of *Angitia* spp. 2 each with 1 specimen of *Apanteles plutellae*
3. P. Appelman	133	33 each with 1 specimen of *Angitia* spp. 19 each with 1 specimen of *Apanteles plutellae* 1 with 2 specimens of *A. plutellae*

Table 19. Field parasitism of first-generation pupae of *Plutella maculipennis* in North Holland, 1939

Sample no.	No. of pupae dissected	Pupae parasitized
1. D. Lighthart	124	1 with 1 specimen of *D. collaris*
2. J. Slaghter	100	31 each with 1 specimen of *D. collaris* 4 each with 2 specimens of *D. collaris* 3 each with 3 specimens of *D. collaris*
3. P. Appelman	100	4 each with 1 specimen of *D. collaris* 1 with 2 specimens of *D. collaris*

Although the percentage parasitism of the host larvae exceeded 40 in all three samples, the only case of superparasitism was that where two larvae of *Apanteles plutellae* occurred in one host: no multiparasitism occurred. In pupae, superparasitism was more frequent although only 38% of the pupae were parasitized in the major sample. In all cases the supernumerary specimens of *Diadromus collaris* had been eliminated in the egg and first larval stages. It is possible that where superparasitism obtained, the parasite eggs were laid within a short time of each other. Thus, in sample 3 of pupae, the superparasitized host contained two eggs. In sample 2, in the hosts containing three parasites, the dead supernumerary specimens were of the same stage—for example, one host contained one living second-instar larva and two dead first-instar larvae of *D. collaris*.

The foregoing data suggest that the difference in oviposition behaviour of these parasites in the laboratory and field is not so great as to render doubtful the utility of laboratory investigations on the lines evolved. The samples are few and the percentage parasitism figures are low, but they support, in a general way, the features of the oviposition responses determined in the laboratory in the preceding winter. Thus, the *Angitia* and *Apanteles* tend to avoid superparasitism and multiparasitism, while *Diadromus collaris* is much more prone to superparasitism. It is unfortunate that further figures for the more heavily parasitized second generation of the host could not be obtained.

6. DISCUSSION

As the present paper deals with the initial work on the interrelations of the parasites of *Plutella maculipennis*, discussion of the data will be brief.

One outstanding feature which emerges from the study of the oviposition behaviour of the present three species of parasites is the extent to which the oviposition response of any one species grades into that of another. Although three very distinct genera are involved, the separation of the factors involving oviposition is not as precise as was anticipated at the outset of the experiments. It might be thought that this latitude in the oviposition responses is largely an expression of the facilities for the females in the laboratory, but the fact that one host species only has been used, and that in states which the adult parasites are liable to meet in the field, somewhat discounts this interpretation. Due caution must be observed in applying laboratory reactions to the natural environment but in some instances there can be little doubt of the identity of the behaviour under the two sets of conditions. For example, it may be taken as certain that *Angitia cerophaga* will oviposit in second-instar *Plutella* larvae [illegible] in prepupae; that *Diadromus collaris* will attack its hosts via the terminal holes of the silken cocoons and lay in prepupae and pupae; and [illegible] these reactions occur in the laboratory [illegible] field. Similarly, the [illegible] experiments indicate an ability to discriminate between parasitized and unparasitized hosts by all three parasites [illegible] there is no reason [illegible] doubt its operation in the field. What the laboratory experiments do not establish, and indeed cannot establish, is the extent [illegible] which this selective faculty determines the incidence of superparasitism and multiparasitism obtaining in a given set of field conditions. Discriminatory faculties seem to be easily countered by other overriding factors in the laboratory and field; so much so that, until queried by Salt in 1934, entomologists had immediately assumed from their observations of the frequency of superparasitism and multiparasitism in the field samples, that the female parasites were unable to distinguish parasitized and unparasitized hosts.

Critics of biological control work frequently decry its empirical nature, but rarely offer a practical alternative method of approach. In the present instance, *Angitia cerophaga* has been established in New Zealand and *Diadromus collaris* was liberated in the 1939 season. Establishment of *Apanteles plutellae* will probably be attempted in later seasons and work on other parasites is proceeding. It is tolerably certain that if the three species under discussion are established, some multiparasitism will occur. Clearly, its extent and importance relative to other useful characters of the parasites can only be accurately assessed in the conditions obtaining in New Zealand; it is difficult to see how it is possible to predict closely the course of such interactions by a study of the behaviour in the laboratory and field in Europe. As H. S. Smith (1929) has pertinently remarked: "Because of the complexity of a parasite's environment, it is doubtful whether one could determine from studies other than actual field observations, just how it would react in the open, and then it would be too late to make use of the knowledge gained." Thus, on the basis of present experimentation, the most that may be claimed is that such competition as might occur between, for example, *Apanteles plutellae* and the established *Angitia cerophaga*, would probably be infrequent on account of the selective faculties of the females of both species. The effect of overriding factors in the form of availability and density of hosts, distribution of the parasites, relative proportion of hosts and parasites in a given area, etc., could only be gauged after liberation of the Braconid species. However, in view of the fact that neither species appears intrinsically superior when both occur in a host, such competition might be regarded as an addition (large or small) to the mortality effects caused by superparasitism in either species.

This question of the relative mortalities in superparasitism and multiparasitism in any two or more species of parasites is one which appears to have been overlooked in the past, although it is an important consideration in gauging the alleged deleterious effects of multiparasitism. Thus Pemberton and Willard (1918) give figures to emphasize the heavy mortality of *Opius humilis* when this species is present in the fruit fly host with *O. tryoni*, but they give no indication of the mortality in cases of superparasitism by *O. humilis*. In one sample of 757 *Ceratitis capitata* collected in the Kona coffee area in 1916, it is shown that 106 cases of duplication of parasitism occurred, in the majority of which a live *Opius tryoni* was found in the same puparium with one or more individual larvae of *O. humilis*. Apparently superparasitism did occur, and the authors remark elsewhere in their paper that they have found as many as ten *O. humilis* larvae in a host capable of maturing one. In his examination of these and other figures

Smith (1929) was therefore doubtful of the ability of *O. humilis* to increase its percentage parasitism of *Ceratitis capitata* since the data suggested that it was not scarcity of parents which determined the percentage parasitism. It is possible, then, that the mortality of *Opius humilis* by superparasitism greatly exceeded that by *O. tryoni* in this district, and the subsequent elimination of *O. humilis* from this part of Hawaii have been as much due to the very high parasitism (and hence superparasitism) which resulted from the easy accessibility of hosts on coffee berries as to the presence of the other two Opiine species. There appears little likelihood of the suppression of *O. humilis* in other host fruits. It has been stated by Thompson (1939) that in the parasite complex of a given pest, superparasitism is generally more frequent than multiparasitism. This excess is usually attributed to the differential effects of environments on the distributions of parasites, but the findings of the foregoing investigation demonstrate that the faculties involved in restricting superparasitism may apply at least equally well to multiparasitism and there is, therefore, the possibility that the oviposition responses of the parasites are such as result in more superparasitism than multiparasitism. This aspect is being examined.

It is, of course, premature to generalize at this stage, but it may be observed that the demonstration in species of the three parasite genera, *Diadromus*, *Angitia* and *Apanteles*, of basic tendencies to avoid parasitizing hosts containing various stages of another species supports H. S. Smith's contention of the *probable* superior effects of introducing several primary parasites of a given pest. Later data on the oviposition behaviour of *Angitia fenestralis* and *A. cerophaga* and *Diadromus subtilicornis* and *D. collaris* may modify this conclusion and will be of particular interest since the responses will be intrageneric and more comparable with the interactions of the three *Opius* species in Hawaii.

Besides their practical bearing, the foregoing experimental data are of general interest from the point of view of insect behaviour. Similar work on host selection by parasites has recently been published by Salt (1935, etc.), Ullyett (1936), and Lloyd (1938). Salt, with *Trichogramma evanescens*, and Lloyd, with *Ooencyrtus kuvanae*, showed that these egg parasites were able to distinguish between parasitized and unparasitized hosts and partially refrain from ovipositing in parasitized hosts. This capacity has now been proved to exist in the Ichneumonid genera *Diadromus* and *Angitia*. Opinion on the nature of the factors involved in the rejection of parasitized hosts, is divided. On the basis of work with *Trichogramma evanescens*, Salt and Laing (1935) claimed that the initial rejection of a parasitized host was due to chemical traces left on the host by the first female and detected by later females and they suggested that a similar response might occur with *Collyria calcitrator* and *Ibalia leucospoides*. The present author (1938) showed the inadequacy of this explanation for *Opius kuvanae*, the egg parasite of the Gipsy Moth, and its operation may be doubted also in the case of *Diadromus collaris* and *Angitia cerophaga*. A full discussion of the behaviour in host selection in the primary parasites of *Plutella maculipennis* is being deferred to a later paper.

The problem was undertaken at the request of the New Zealand Entomologists, Dr Miller and Mr Muggeridge, to whom the writer is indebted for the suggestion. The work was carried out under the supervision of Dr W. R. Thompson, F.R.S., whose interest and criticism throughout have been most helpful.

7. SUMMARY

1. An investigation is being made of the incidence in the laboratory and field of superparasitism and multiparasitism in the primary parasites of *Plutella maculipennis* Curt. The present paper deals mainly with laboratory experimental work on the three species *Diadromus collaris* Grav., *Angitia cerophaga* Grav. and *Apanteles plutellae* Kurdj.
2. From the experimental point of view, the problem of multiple parasitism is being regarded as a question of the nature of the oviposition responses of the various parasites. The procedure adopted consists in confining the female parasites in tubes for a fixed time with a given number of hosts and observing the oviposition behaviour of the adult parasites and the subsequent development of the immature parasites, to note the effects of competition.
3. With *Diadromus collaris* the silken cocoon surrounding the pupa is important in securing maximum oviposition because of the posture adopted by the female in the oviposition act. Unparasitized hosts were more acceptable when histogenesis was in the early stages but hosts parasitized in such stages were usually rejected by subsequent females. This rejection was very well defined in respect of pupae parasitized by *D. collaris* itself or prepupae containing advanced stages of *Angitia cerophaga* and was exercised when such hosts were given to the females for numerous brief exposures. Superparasitism in pupae and prepupae occurred only in cases where the host contained recently deposited eggs; multiparasitism obtained when the *Diadromus* female encountered prepupae containing early instars of *Angitia cerophaga*, but oviposition in these conditions was restricted.

Experiments suggest that feeding in host contents is necessary for the full development of the gonads.

4. The parasite *Angitia cerophaga* will attack all host larval instars and also occasionally prepupae in cocoons. Larvae containing eggs or first and second instars of *A. cerophaga* were usually rejected when unparasitized hosts were available; when parasitized hosts only were given to the females oviposition was limited. An ability to distinguish between unparasitized host larvae and those containing eggs of *Apanteles plutellae* was also observed.

5. *Apanteles plutellae* oviposited in the first three host larval instars and tended to avoid parasitizing hosts containing immature stages of its own species or those of *Angitia cerophaga*.

6. Elimination of supernumerary larvae in superparasitism in *Diadromus collaris* was usually by combat in the first and second instars. When present in a prepupa with an early instar of *Angitia cerophaga*, neither species survived. Suppression of excess larvae in superparasitism in *Angitia cerophaga* and *Apanteles plutellae* was by physiological inhibition in the first instar. This also occurred when specimens of both species were found in one host, but neither species appeared intrinsically superior if the eggs were laid within a short interval.

7. Some data on field parasitism in the first generation of the host in North Holland in 1939 are included. The details for superparasitism and multiparasitism support laboratory experimental work.

8. The relation of the findings to biological control practice of introducing several primary parasites of a given pest is briefly examined.

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The paedogenetic male cycle in *Salmo salar* L.

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Introduction and literature

In a paper in 1938 we, along with G. M. King, gave an account of the ripe male sexual phase in salmon parr from the Welsh Dee. In this paper it was shown that the attainment of the ripe male phase is normal in at least a high percentage (about 80%) of the male population, and not merely in a few individuals. This work was complementary to that of Carr (1913), Dahl (1910) and others (vide Tchernavin 1939), and extended and clarified their observations. It was also shown that among migrating fish (smolts) there were (1) males with apparently spent gonads, (2) the normal females with immature gonads, and (3) individuals with tubular gonads extending the length of the body cavity. Some doubt was felt about the sex of fish with long tubular gonads, but they were thought to be and have now been proved to be males, thus confirming Carr's assumption (*loc. cit.*). Most of these tubular gonads are immature and virgin.

The problem remained whether in fact young males spawn on the beds before migrating to the sea. It was noted that many statements in the literature affirmed that this occurred, but no critical observations appear to have been made. At the present time we consider that the only scientific evidence we have on this matter is circumstantial and is supplied in the observations recorded below.

There is a considerable salmon literature widely dispersed in a variety of publications, and we are indebted to Tchernavin for a recent valuable review (1939). So far as we have been able to examine it, we have been unable to find any adequate critical examination of the post-spawning and smolt gonad phases in the life-history of the young male salmon. It is true that isolated shrewd observations and suggestions have been made. Some of these are proved and substantiated in this paper, the object of which is to present data that in themselves may reveal phases in the life history. There is no doubt that difficulty in obtaining adequate quantities of material, and its capture mainly by rod and line, demands circumspection in interpreting the data.

The age estimations from the scales and the investigations on the histology of the testis have all been made by one of us (J. W. J.).

PHASES OF THE GONAD IN PARR AND SMOLTS

Some 2790 parr and smolts have been examined from English and Welsh rivers; of these 1389 (50 %) were males. Most of them were taken by rod and line, only 540 being netted. The bulk of the males, 766, were obtained from the Welsh and Cheshire Dee, 220 came from the Wnion in North Wales, and the remainder in small numbers from fourteen English and Welsh rivers; full particulars of all samples are given in tables 2 and 3.

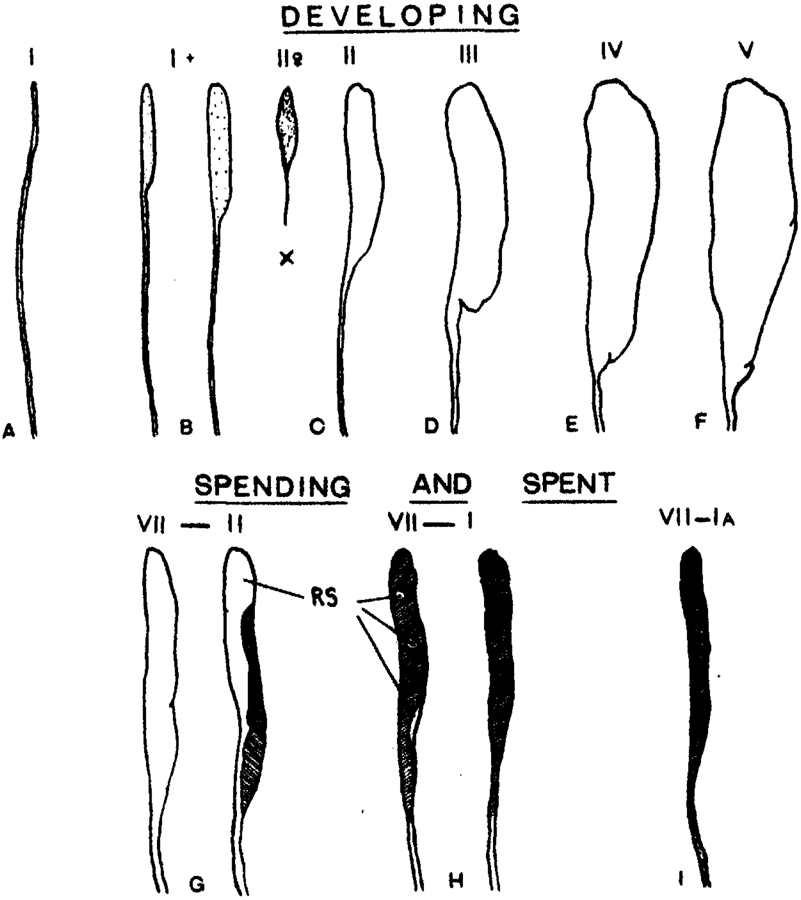

FIGURE 1. Gonad phases in salmon parr and smolts. Patches of relict sperm (*R.S.*) are to be seen in *H*, the spent testis. Sperm is represented in all phases by the white (unshaded) colour.

The gonad phases of all fish were examined and recorded in accordance with the scheme of nomenclature recommended by the International

Council. These phases as applied to young salmon are shown in figure 1 and were given in our earlier paper (1938, p. 107).

(*a*) *Ovary*

All the females examined had the gonad in stage II (see figure 1 x). This is a resting stage in which eggs are developed up to a size of about 250 μ in diameter. It is acquired in yearling salmon, and its relative size showed no appreciable change in any of the fish. Arwiddson (1910) gives further information on this stage. It is clear that under exceptional conditions the female gonad may mature and produce ripe ova in fresh water (Regan 1938), but such conditions rarely occur in the normal habitat in rivers.

(*b*) *Testis*

Throughout the year the testis may occur in one or other of the phases shown in figure 1. When development (stages II–V) takes place it begins in the summer and maturity (stage VI) is attained in the autumn. Running fish (stage VI), namely, individuals ready to spawn, occur in the Welsh Dee towards the end of September and can be found until December with a maximum about November. From November to May spent fish (stage VII) occur among parr and smolts, and the cycle is begun again in some of the fish remaining in the river soon after the smolts migrate.

The occurrence and frequency of the various stages at different ages are given in table 1 and are shown in figure 2. The samples received are graphed arbitrarily in the following periods:

(1) June–July; beginning of development of the testis.
(2) August, September, October; completion of development.
(3) November–December; spawning phases.
(4) January–May; spent (partially or completely) and immature phases.
(5) Smolts only; spent and immature phases.

Excepting the smolts most of the fish are in their second summer of growth, that is, 1+ years becoming 2 years in October. Relatively few 0-group and 3-year individuals were captured. The mode of capture, chiefly by rod and line, undoubtedly selected the larger fish amongst those which would feed.

In June and July (figure 2, graph 1) the gonads show early development to stages II and III. There are a few spent stages of individuals which did not migrate. During August to October (graph 2) gonad development is completed and the bulk of the population have testes in stages III to VI. At the end of this period the fish aged 1+ become age 2 fish as at X; the two ages are shown separately for this period. A fair number of fish 1+ and 2 years old and an

TABLE 1. NUMBER AND PERCENTAGE OF MALE GONAD PHASES OF SALMON PARR AND SMOLTS IN EACH AGE-GROUP FOR SEASONAL PERIODS

Groups Gonad Stage ...	I and I+			II			III and IV				V and VI				Spending or spent			? Spent		
Age ...	1	2	3	1	2	3	1	2	2+	3	1	2	2+	3	1	2	3	1	2	3
June–July	112	3	—	32	15	1	12	10	—	2	—	—	—	—	—	1	1	9	8	—
%	54·4	1·5	—	15·5	7·3	0·5	5·8	4·9	—	1·0	—	—	—	—	—	0·5	0·5	4·4	3·9	—
Aug.–Sept.–Oct.	15	46	1	1	—	—	17	52	8	13	7	35	8	12	—	—	—	—	3	—
%	6·9	21·1	0·5	0·5	—	—	7·8	23·9	3·7	6·0	3·2	16·1	3·7	5·5	—	—	—	—	1·4	—
Nov.–Dec.	—	16	—	—	—	—	1	2	—	—	—	3	—	—	—	33	7	—	—	—
%	—	25·8	—	—	—	—	1·6	3·2	—	—	—	4·8	—	—	—	53·3	11·3	—	—	—
Jan.–May	19	143	3	—	—	—	—	—	—	—	—	—	—	—	—	105	42	—	8	2
%	5·9	44·4	0·9	—	—	—	—	—	—	—	—	—	—	—	—	32·6	13·0	—	2·5	0·6
Smolts	49	141	3	—	—	—	—	—	—	—	—	—	—	—	13	133	19*	15	93	7
%	10·4	29·8	0·6	—	—	—	—	—	—	—	—	—	—	—	2·7	28·1	4·0	3·2	19·7	1·5

* In addition one four-year-old fish occurred amongst the smolts (=0·2 % of the total males).

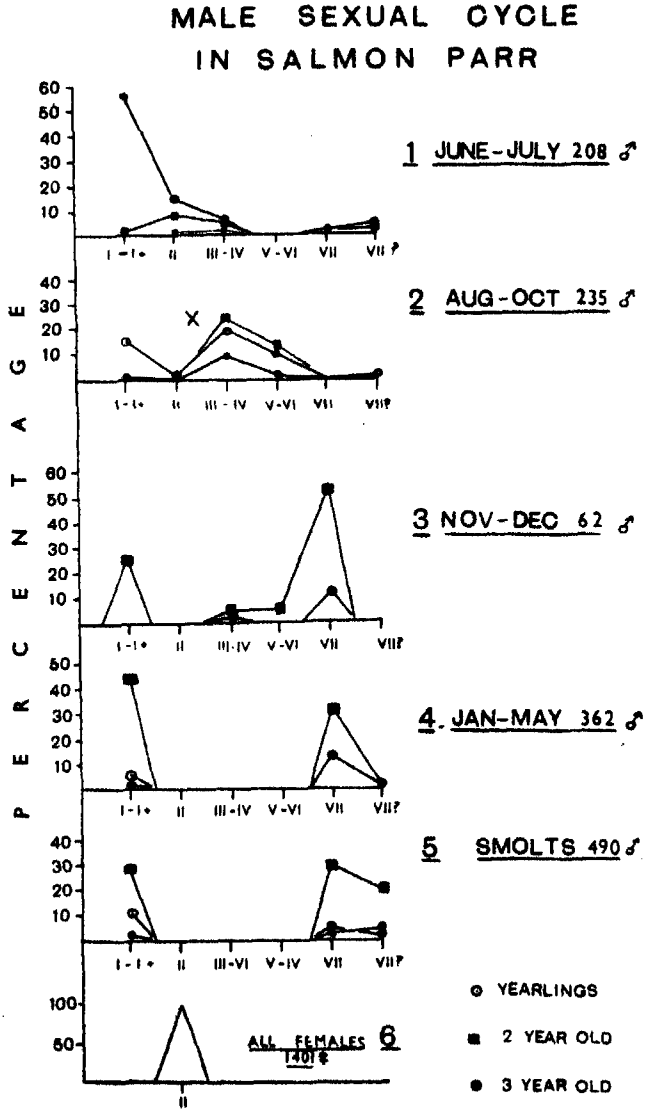

Figure 2. Percentage frequency of gonad phases with age in (1–5) male salmon parr and smolts at different periods of the annual sexual cycle. (English and Welsh rivers.) (1) Beginning and (2) completion of the period of gonad development. (3) Spawning period. (4) Post-spawning period mainly and (5) migrating period (smolts). (6) Gives the uniform immature condition in all females.

Table 2. Sex phases in samples of salmon parr and smolts from the Welsh Dee

River and date		Female	Male gonad phases						
			I and I+	II	III–IV	V–VI	Spent	Spent ?	Re-covering
Ceiriog	14. ii. 38	7	1	—	—	—	6	—	—
Ceiriog	15. ii. 38	5	8	—	—	—	8	—	—
Ceiriog	22. ii. 38	8	8	—	—	—	5	—	—
Dee	24. ii. 38	11	9	—	—	—	7	—	—
Dee	(*a*) 25. ii. 38	15	9	—	—	—	1	—	—
Dee	(*b*) 25. ii. 38	16	7	—	—	—	2	—	—
Llandrillo Brook	1. iii. 38	6	4	—	—	—	7	—	—
Dee	2. iii. 38	7	3	—	—	—	6	—	—
Alwen	3. iii. 38	4	8	—	—	—	2	1	—
Dee	4. iii. 38	5	3	—	—	—	1	2	—
Alwen	7. iii. 38	20	5	—	—	—	6	—	—
Dee	8. iii. 38	3	3	—	—	—	1	2	—
Alwen	8. iii. 38	3	4	—	—	—	5	—	—
Alwen	9. iii. 38	4	7	—	—	—	3	—	—
Dee	10. iii. 38	8	8	—	—	—	6	—	—
Dee	11. iii. 38	9	6	—	—	—	1	—	—
Dee	14. iii. 38	9	4	—	—	—	—	—	—
Alwen	14. iii. 38	15	6	—	—	—	9	—	—
Alwen	15. iii. 38	4	3	—	—	—	5	—	—
Dee	17. iii. 38	13	11	—	—	—	—	—	—
Dee	(*a*) 20. iv. 38	6	5	—	—	—	—	1	—
Dee	(*b*) 20. iv. 38	6	1	—	—	—	—	1	—
*Smolts	26. iv. 38	16	5	—	—	—	1	2	—
*Smolts	29. iv. 38	9	—	—	—	—	2	5	—
*Smolts	9. v. 38	26	18	—	—	—	4	5	—
*Smolts	12. v. 38	39	15	—	—	—	9	8	—
Non smolts	13. v. 38	20	5	—	—	—	—	—	—
*Smolts	17. v. 38	32	7	—	—	—	2	9	—
*Smolts	25. v. 38	23	23	—	—	—	7	—	—
Dee	9. vii. 38	6	2	—	—	—	—	1	—
Dee	12. vii. 38	4	—	5	3	—	—	4	—
Alwen	14. vii. 38	5	5	7	—	—	—	—	—
Ceiriog	16. vii. 38	8	7	3	9	—	—	—	—
Alwen	27. vii. 38	3	—	2	3	—	—	—	—
Dee	(*a*) 27. vii. 38	1	1	4	2	—	—	—	—
Dee	(*b*) 27. vii. 38	6	3	—	—	—	—	—	—
Ceiriog	27. vii. 38	5	1	2	1	—	—	—	—
Ceiriog	15. viii. 38	4	1	—	4	—	—	—	—
Dee	(*a*) 15. viii. 38	3	4	—	—	—	—	—	—
Dee	(*b*) 15. viii. 38	2	—	—	4	—	—	—	—
Alwen	15. viii. 38	—	1	—	5	—	—	—	—
Ceiriog	20. ix. 38	4	—	—	4	—	—	—	—
Dee	20. ix. 38	5	4	—	—	—	—	—	—
Alwen	20. ix. 38	4	—	—	4	—	—	—	—

* Taken by hand-net at Chester Weir.

TABLE 2 (*continued*)

River and date		Female	Male gonad phases						
			I and I+	II	III–IV	V–VI	Spent	Spent ?	Re-covering
Llandrillo Brook	15. x. 38	2	1	—	3	2	—	—	—
Alwen	15. x. 38	—	—	—	6	2	—	—	—
Ceiriog	19. x. 38	5	—	—	3	—	—	—	—
Alwen	8. xi. 38	4	—	—	—	2	—	—	—
Dee	20. xii. 38	1	—	—	—	—	3	—	—
Ceiriog	20. xii. 38	2	—	—	—	—	6	—	—
Ceiriog	29. xii. 38	3	1	—	—	—	4	—	—
Dee	29. xii. 38	2	1	—	—	—	5	—	—
Alwen	30. xii. 38	1	—	—	—	—	7	—	—
Alwen	24. i. 39	1	—	—	—	—	5	—	—
Alwen	28. i. 39	1	—	—	—	—	2	—	—
Ceiriog	31. i. 39	5	—	—	—	—	5	—	—
Dee	31. i. 39	3	—	—	—	—	5	—	—
Alwen	21. ii. 39	2	3	—	—	—	5	—	—
Dee	22. ii. 39	7	1	—	—	—	3	—	—
Ceiriog	22. ii. 39	6	—	—	—	—	4	—	—
Dee	14. iii. 39	3	4	—	—	—	1	2	—
Alwen	15. iii. 39	1	1	—	—	—	8	—	—
Ceiriog	15. iii. 39	6	1	—	—	—	4	—	—
Dee	14. iv. 39	7	2	—	—	—	3	—	—
Alwen	14. iv. 39	2	2	—	—	—	6	—	—
Ceiriog	14. iv. 39	7	1	—	—	—	2	—	—
Ceiriog	16. v. 39	3	—	—	—	—	1	5	—
Dee	16. v. 39	5	7	—	—	—	—	—	—
*Smolts	19. v. 39	15	2	—	—	—	5	2	—
Dee	29. v. 39	3	2	—	—	—	2	1	—
Alwen	1. vi. 39	3	3	—	—	—	2	2	—
Alwen	7. vi. 39	6	2	—	—	—	—	2	—
Ceiriog	13. vi. 39	3	2	—	—	—	—	5	—
Dee	13. vi. 39	4	6	—	—	—	—	—	—
Dee	27. vi. 39	9	9	1	—	—	—	2	2
Ceiriog	11. vii. 39	4	—	5	1	—	—	—	—
Dee	12. vii. 39	4	3	3	—	—	—	—	—
Alwen	12. vii. 39	4	4	1	1	—	—	—	—
Dee	12. viii. 39	7	3	—	—	—	—	—	—
Ceiriog	12. viii. 39	4	1	3	2	1	—	—	—
Alwen	12. viii. 39	3	1	—	5	1	—	—	—
Ceiriog	20. ix. 39	3	1	—	6	2	—	—	—
Alwen	20. ix. 39	1	3	—	2	4	—	—	—
Dee	20. ix. 39	7	4	—	—	1	—	—	—
	Totals	568	296	36	68	15	194	62	2

* Taken by hand-net at Chester Weir.

occasional 3-year-old have immature testes. There are occasional doubtful spent testes still to be found. In November–December (graph 3) a large proportion of the fish are either spent or in late stages of maturity.

In the five months from January to May (figure 2, graph 4) the population is now composed entirely of immature and spent fish (see also Jones 1940).

The condition among the migrating fish, the smolts (figure 1, graph 5), is essentially the same as in the post-spawning parr population (period January to May) of which it is virtually a sample. The occurrence among the smolts of only immature or spent gonads, in samples from sixteen different rivers, is undoubtedly significant; but it may be noted that the testes would be of two sizes, as the stage I tubular gonad is smaller than the spent stage VII–II or VII–I. It is possible that this may account for the analysis of salmon smolt gonads made by Pentelow and others (1933).

It is virtually certain from the data given in table 1 and figure 2 that the male population of salmon parr exhibit a sexual cycle, in which the gonad develops in summer, reaches maturity in autumn, and becomes spent in the winter; also that after spawning a high percentage of the males migrate to sea. All the 490 male smolts had quiescent testes, either spent or immature. An account of the histological changes in the sexual cycle in the gonad in male salmon parr is given by one of us (Jones 1940).

The disappearance from the samples of males with full gonads after the spawning season round about November, when the large females make most of the redds, offers circumstantial evidence that the young males have spawned in the river. There is no clear evidence as yet that these young males spawn with the large immigrating females, but the provision of such evidence either by experiment or critical observation may be expected in the near future.

HISTOLOGY OF THE MALE GONAD PHASES

A histological examination of the male gonad phases (Jones 1940) has shown that all testes recorded as stage I (tubes) (see figure 1) were immature. Spermatogenetic development is correlated with a widening of the testes anteriorly (1+ stages), and as spermatogenesis proceeds the testes gradually fill with sperm, encroach posteriorly and eventually fill up the body cavity (stages V–VI). Spawning results in a general collapse of the gonads which brings about a thickening of the tunica and crypt walls. The macroscopic changes brought about by the ripening and the spending have been described. The structure of the spent testis confirms the naked-eye appearance and renders identification easy in the freshly killed fish. There is no doubt that fishes with spent gonads have spawned in the river.

RIVER TEMPERATURE DURING THE PERIOD OF GONAD DEVELOPMENT

Gonad development in salmon parr in the localities studied begins about the middle of May and is completed about the end of October or earlier. There are indications that this development may begin somewhat earlier in the warmer rivers. It is therefore probable that temperature is one of the more important factors controlling gonad development. In the Dee the temperature during the approximate period of gonad development (middle of May to end of September) is maintained above a level of about 52° F (see figure 3). It would be interesting to know if such a correlation is constant throughout the geographical range of this fish. This may very well be so, as breeding in many poikilothermous animals is known to be associated with particular temperature levels or accelerated temperature changes (Turner 1919; Orton 1920).

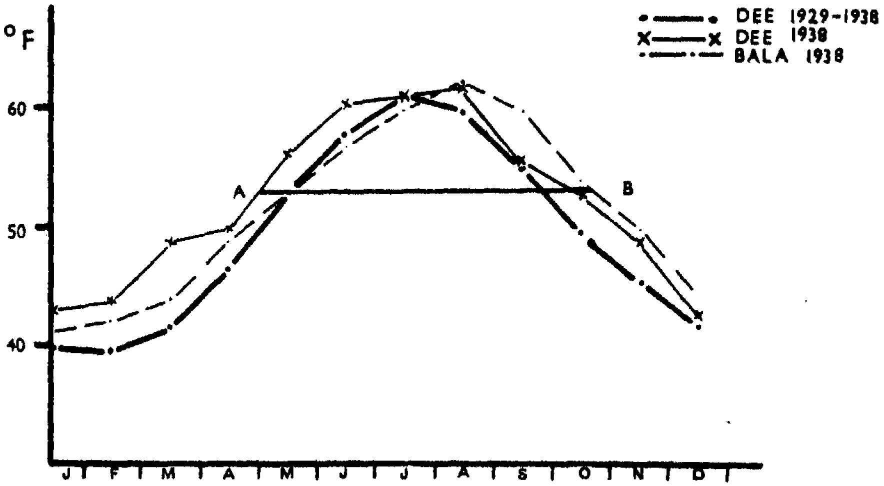

FIGURE 3. Seasonal variation in temperature of the River Dee (Wales and Cheshire). Gonad development occurs during the period *A–B* and in the temperature range shown above the line *AB*.

The discontinuous thick line gives monthly means of daily readings recorded for ten years by the Dee Fishery Board (*Reports* 1933, 1938), the thin line with crosses gives the monthly means from the same source for the Dee for 1938. The thin discontinuous line gives monthly means of regular daily records taken with an accurate Calderara sea surface thermometer (N.P.L. certificate) at the overflow of Bala Lake into, and the source of, the main Dee.

TABLE 3. SEX PHASES IN SALMON PARR AND SMOLTS FROM ENGLISH* AND WELSH RIVERS OTHER THAN THE WELSH DEE

			Male gonad phases					
River and date		Female	I and I+	II	III–IV	V–VI	Spent	Spent ?
Fowey	30. iii. 38	1	—	—	—	—	—	—
Wnion	30. iii. 38	1	—	—	—	—	1	—
Dart	7. iv. 38	7	7	—	—	—	—	—
Wnion	7. iv. 38	3	1	—	—	—	1	—
Taw	9. iv. 38	17	11	—	—	—	1	1
Taw	9. iv. 38	14	—	—	—	—	3	7
Yarty	20. iv. 38	—	—	—	—	—	1	—
Wnion	22. iv. 38	8	—	—	—	—	3	3
Wnion	26. iv. 38	34	—	—	—	—	1	4
Derwent	29. iv. 38	30	6	—	—	—	4	5
Dart	30. iv. 38	9	—	—	—	—	—	4
Wnion	3. v. 38	11	3	—	—	—	3	9
Ure (Yore)	3. v. 38	11	4	—	—	—	9	3
Ruswarp	6. v. 38	9	1	—	—	—	2	2
Wnion	9. v. 38	7	4	—	—	—	7	—
†Derwent	12. v. 38	31	4	—	—	—	6	9
Severn	14. v. 38	5	4	—	—	—	3	—
Ure (Yore)	14. v. 38	13	4	—	—	—	4	3
Glaisdale	17. v. 38	4	—	—	—	—	2	—
Tyne	17. v. 38	17	4	—	—	—	3	—
Severn	20. v. 38	8	4	—	—	—	—	—
Seiont	27. v. 38	2	2	—	—	—	6	—
Elwy	30. v. 38	—	—	—	—	—	1	—
Teme	8. vi. 38	—	1	—	—	—	1	—
Tees	14. vi. 38	—	—	—	—	—	3	—
Seiont	17. vi. 38	—	—	2	—	—	2	2
Wnion	18. vi. 38	7	—	—	—	—	—	5
Eden	20. vi. 38	—	—	—	—	—	1	—
Teme	2. vii. 38	1	—	—	—	—	—	1
Wnion	4. vii. 38	4	1	1	—	—	—	5
Wnion	6. vii. 38	5	—	4	—	—	—	3
Teme	17. vii. 38	—	—	2	—	—	—	—
Wnion	28. vii. 38	4	—	—	2	—	—	—
Teme	29. vii. 38	3	—	2	—	—	—	—
Wnion	26. viii. 38	3	—	1	2	1	—	—
Eden	30. viii. 38	4	1	—	1	—	—	—
Wnion	6. ix. 38	6	—	1	4	—	—	—
Teme	14. ix. 38	1	—	—	2	—	—	—
Teme	20. ix. 38	1	—	—	1	—	—	—
Teme	24. ix. 38	1	—	—	—	2	—	—
Teme	27. ix. 38	—	—	—	1	—	—	—
Wnion	13. x. 38	1	—	—	2	—	—	—

* Samples included from the R. Forss, N. Scotland.

† Netted samples.

TABLE 3 (*continued*)

River and date		Female	Male gonad phases					
			I and I+	II	III–IV	V–VI	Spent	Spent ?
Wnion	20. x. 38	3	1	—	2	—	—	—
Wnion	29. x. 38	1	—	—	1	—	—	—
Wnion	9. xi. 38	2	—	—	2	1	—	—
Wnion	18. xi. 38	2	—	—	—	—	5	—
Conway	19. xi. 38	19	5	—	—	—	5	—
Wnion	23. xi. 38	9	1	—	—	—	2	—
Wnion	2. xii. 38	2	—	—	—	—	2	—
Wnion	15. xii. 38	3	—	—	—	—	1	—
Wnion	22. ii. 39	4	—	—	—	—	2	—
Wnion	25. ii. 39	2	—	—	—	—	—	—
Wnion	10. iii. 39	3	—	—	—	—	1	—
Wnion	11. iii. 39	—	2	—	—	—	3	—
Wnion	30. iii. 39	2	2	—	—	—	1	—
*Wnion	30. iii. 39	12	10	—	—	—	12	2
Wnion	4. iv. 39	4	2	—	—	—	—	—
Wnion	12. iv. 39	2	—	—	—	—	3	—
*Eden	12. iv. 39	28	4	—	—	—	5	3
Wnion	20. iv. 39	37	4	—	—	—	11	—
Wnion	26. iv. 39	11	—	—	—	—	1	—
Wnion	28. iv. 39	8	—	—	—	—	2	—
*Eden	5. v. 39	38	15	—	—	—	15	6
Wnion	9. v. 39	3	1	—	—	—	5	2
Tyne	10. v. 39	29	8	—	—	—	6	4
Wnion	16. v. 39	8	1	—	—	—	3	—
Tyne	20. v. 39	30	3	—	—	—	8	6
Wnion	24. v. 39	1	3	—	—	—	—	1
North Esk	24. v. 39	11	9	—	—	—	1	3
Forss	26. v. 39	20	1	—	—	—	11	4
*Wnion	5. vi. 39	51	41	3	—	—	—	—
Wnion	8. vi. 39	—	1	—	—	—	—	1
Wnion	21. vi. 39	6	4	—	—	—	2	—
Wnion	8. vii. 39	3	1	—	—	—	—	—
Wnion	14. vii. 39	8	9	3	—	—	—	—
Forss	18. vii. 39	17	13	3	—	—	—	—
Forss	25. vii. 39	25	10	7	12	2	—	—
	Totals	687	213	29	32	6	175	98

* Netted samples.

SPAWNING OF MALES IN SUCCESSIVE YEARS

Testes of the stage VII–II or VII–I type have been found exhibiting signs of redevelopment as shown by an abundance of germ cells in the crypts, but also having a thick tunica; further information on this kind of gonad is given by Jones (1940). This type has not been fully investigated

but we have little doubt that at least some few males spawn in successive years in the river. Hoek (1910) has suggested that this might be so.

TWO TYPES OF MALE SALMON SMOLT

The analysis of male sex phases given in figure 1, graph 5, gives a picture of the condition in male smolts. Some 40% have immature gonads and the remainder mostly gonads which have functioned. It is therefore a fact that two different kinds of males migrate to the sea from the river. Whether these are genetic or physiological types remains to be found in the future. The proportions in which these two types occur in our samples may not be representative of the migrating fish in any one river, as they are in the first place chiefly rod-caught fish, and in the second combined totals for all rivers.

The difference in the physiological condition of the two types may very well be reflected in their behaviour in the sea and in the date of their return to the river to spawn again. The proportions of the two types of males leaving a river in a particular year may be related to the variations found in the annual proportion of grilse returning to rivers and the high proportion of males among grilse (Menzies 1931; Hutton 1938).

It is highly desirable that the proportions of the two types of males should be known, and in the first instance for all the more important salmon rivers. The suggestion that two kinds of adult male salmon occur may be found in salmon literature.

During the forthcoming investigations on salmon biology in the River Forss the samples of parr and smolts collected for various purposes will also be examined for sex and sex proportions. It is anticipated that netted samples* will be available for a thorough study of the obscure points in the paedogenetic male cycle.

CHARACTER OF SCALES IN MATURING MALE PARR

The scales of all the parr and smolts have been investigated and are described elsewhere (Jones 1939). The problem of special interest is whether maturing male parr have summer rings on their scales and are therefore presumably growing while developing the gonad.

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* Only three samples were obtained and are recorded in table 3.

The older male parr in British rivers

The occurrence of three-year-old males among the spent categories confirms the work of many workers. Only one four-year-old male has been encountered in the English and Welsh rivers dealt with. Four-, five- and six-year-old parr or smolts are known from Sweden (Dahl 1910).

Mortality among spent parr

Our material is not extensive enough to permit definite statements on the matter of mortality among spawned parr. Among the population at the beginning of the spawning season, i.e. November–December, 75% of the male parr have been found to be sexually ripe or nearly so. As this population mostly migrates in the following April to June as smolts, the proportion of spent and ripe males amongst these smolts might be expected to be about the same as in November–December. Actually only 60% of the male smolts gave evidence—as spents—of having functioned. The discrepancy, 15%, may be an indication that a small post-spawning mortality has occurred, but as an unknown proportion of males may remain in the river to spawn again as parr [see figure 2 (1)], it is doubtful whether the differences observed are significant. Further doubt arises from the selective method of fishing and the small number, 62, taken in the November–December period. It would appear, however, that the post-spawning mortality among male parr cannot be great.

Generality of the paedogenetic cycle in the Atlantic male salmon throughout its distribution

From the accounts given in the literature of apparently occasional ripe salmon parr, and in other cases of the observance of larger numbers of ripe parr, there can remain no doubt that paedogenesis in the male occurs throughout the geographical range of this species. The proportions and age of mature young males in different localities is not sufficiently well known. Knowledge of these factors in different localities correlated with general environmental factors, and with the age and sex constitution of the adult population, may very well add much information to the biology of this interesting fish.

Hutton's analysis of sex proportions among adult salmon for the Wye (1938) shows a remarkable predominance of females, especially among the younger groups. If these observations are confirmed and hold for other rivers, it would appear that the relative paucity of adult males may be compensated for biologically by the abundant paedogenetic males.

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SUMMARY

In a further study of the male sex phases throughout the year in salmon parr—accompanied by histological investigation of the gonad—the earlier observations have been confirmed. Some 2790 parr and smolts, captured mainly by rod and line from English and Welsh rivers, have been examined; 50 % of these were male. All the females are immature virgins; the males exhibit a seasonal sexual cycle in which the gonad develops in the summer, becomes mature in the autumn and is spent in winter. After spawning, a high proportion of the male parr migrate to sea as smolts. All the male smolts examined, i.e. 490, had quiescent testes, being either spent, or immature and virgin. It is hereby established that two types of male smolts migrate to sea. The gradual disappearance of ripe male parr during the spawning season (beginning about November) and the correlated appearance and increase of males with spent gonads renders it virtually certain that the ripe male parr spawn on the salmon redds.

The temperature range in the rivers during the development of the male gonad is noted.

The finding of only 60 % spent amongst the migrating smolt males as against 75 % ripe, spending and spent male parr in the preceding spawning period, November and December, may be an indication of a slight mortality among the spawning males.

The sex records given herein, along with those of ripe parr in the literature, leave no doubt that paedogenesis in the male of the Atlantic salmon *Salmo salar* L. occurs throughout its geographical range.

The remarkable paucity of males amongst the younger classes of immigrating salmon noted by Hutton on the Wye may be general, and be compensated by the abundant paedogenetic males.

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Histological changes in the testis in the sexual cycle of male salmon parr (*Salmo salar* L. juv.)

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[Plates 20–22]

INTRODUCTION

A histological examination of the parr testis was begun in conjunction with an investigation of the occurrence of the male sexual stage in salmon parr (Orton, Jones and King 1938; Jones and Orton 1940). It became apparent that histological preparations were necessary in order to ascertain definitely whether testes which had been classified as "spent" or "empty" belonged to fish that had spawned, and whether it was possible to distinguish by macroscopic examination between an undeveloped and a spent testis. Subsequently it was considered desirable to follow out histologically the complete cycle from the earliest stage sampled to the spent condition and after.

The fish examined were mostly killed on the riverside and the material fixed in Bouin's fluid. Some formalin material was also used. After cutting at about 5–7μ the sections were stained with Ehrlich's haematoxylin or Heidenhain's iron haematoxylin, both being counterstained with eosin. Staining with Mallory's triple stain proved unsuccessful.

The amount of work done on the sexual cycle of fishes is not great. The most detailed account is that by C. L. Turner on the seasonal cycle in the perch spermary. As will be seen later, this account makes possible a very thorough comparison between the sexual cycles of perch and salmon. Other work has been done on lamprey (Cunningham 1887), Salmonidae (Felix 1897), *Raja* (Beard 1900), *Acanthias* (Woods 1902), trout (Federow 1907), *Lophius* (Dodds 1910), *Lepidosiren* (Agar 1911), perch (Turner 1919), trout (Mrsïc 1923), *Umbra* (Foley 1921 and 1926), *Fundulus* (Matthews 1938), *Brachyraphis* (Turner 1938).

1. GENERAL REVIEW OF THE SEXUAL CYCLE OF MALE PARR

The testes are paired bodies situated dorsal to the gut and ventrolateral to the air bladder. They are unconnected along their whole length. The youngest testes sampled were thread-like bodies of an even grey colour which in some cases was slightly translucent (see figure 1 A, Orton and Jones 1940). Such testes were found throughout the year in all sizes of fish, but more commonly in the smaller fish, and were classified as the stage I tube type characteristic of non-developing gonads.

As development progresses these testes widen anteriorly, often taking on a slightly rosy colour, but no changes take place posteriorly; the posterior part remains thread-like, and for the sake of convenience is termed the duct. Testes showing a definite widening anteriorly were classified as I+ (figure 1 B, Orton and Jones, *loc. cit.*). These will definitely mature during the succeeding three or four months. As development proceeds, the anterior widening extends posteriorly and a gradual flattening occurs. The colour of the testicular portion remains rosy, and of the duct, grey. A well-developed blood vessel is to be seen extending along the inner and thicker portion of the testes.

When the testes are about 5 mm. wide (stage II, figure 1 C, Orton and Jones, *loc. cit.*), the testicular portion may extend to about half the length of the body cavity. There occurs a gradual change of colour from the rosy tint to a light grey, which eventually will give place to a greasy white, the latter colour appearing first at the anterior end. Intermediate colour stages occur, some being a dark rosy purple posteriorly but greasy white anteriorly.

As development proceeds the testes fill and the wider anterior part encroaches posteriorly. Consequently, the free part of the duct is shortened, but even the narrowest part of the duct is at this stage considerably broader than the duct of the stage I testes. When the testes fill about half the body cavity they are classified as stage III (figure 1 D, Orton and Jones, *loc. cit.*). Testes in this stage have gradually lost their greasy white colour and are more of a milky white. Figure 1 E shows stage IV which fills about two-thirds of the body cavity, and 1 F, stages V and VI filling almost all available space. Stage VI differs from V in having ducts full of sperm oozing to the exterior.

During spawning the testes begin to collapse and revert to sizes similar to those of the developmental stages above. However, they differ from the developmental forms, primarily in the state of the duct, which is often still full of sperm or clumps of sperm that may ooze to the exterior on slight pressure of the abdomen. Spent or partly spent testes were recorded as VII–IV, VII–III, VII–II, VII–I+, etc., the second numeral designating the size of the collapsing testes in comparison with the developing forms of that stage. It is not until stages VII–II and VII–I+ are reached that pronounced changes occur, these testes being much collapsed with parallel sides (figure 1 G–I, Orton and Jones 1940) and often subcylindrical in cross-section. When the testis is reduced to stage VII–II, it acquires a rosy colour which appears in patches and spreads posteriorly until only the ducts remain white. Patches of relict sperm form white spots and are often accompanied by a browny orange colour, which in turn becomes peachy. A peachy colour, with occasional spots of relict sperm, and with sperm in the duct, are characteristic of a spent testis. Eventually the relict sperm disappears and the duct empties, the longitudinal and transverse blood vessels stand out, and the duct becomes translucent. A further collapse of the testis is associated with longitudinal folds on the testicular portion. Continued flattening and narrowing of the testes and a collapse of the ducts is also associated with a further change in colour, the peachy colour being superseded by a brownish pink, which will eventually become an even fawny colour. The testes in the fawny stage are long and narrow, and no further stages in the spending can be recognized definitely macroscopically.

2. THE SEASONAL CYCLE

The early stage I was present in samples taken at all times of the year. The cycle may be advantageously followed in the fish remaining in the river after a smolt migration. The first sample of such category was not taken until 9 July 1938, from the Dee. Spent gonads were rarely present, but others were developing. These I+ testes were widening anteriorly where the greyish colour had already superseded the rosy colour still dominant posteriorly. Some indeterminate testes which may have been either spent or redeveloping were recorded. A sample received five days later contained a preponderance of developing testes in stages I+, II, II+ and III. Of the III's one was almost pure white, but the other less advanced, being a dark purplish red posteriorly but white anteriorly. The word 'stage' is often deliberately omitted as here for convenience.

Towards the end of July of the twenty-seven fish taken from the Ceiriog six of the nineteen males present were still I+, but several stage III's were seen. The first stage IV was recorded from the Alwen on 27 July. It is interesting to note that the Dee sample near Corwen taken on the same date contained only III's and II's, whereas a contemporaneous sample from lower down the Dee, near Newbridge, contained only I+ forms. During August, samples were received on the 15th; those from the Alwen and Ceiriog contained advanced stages but the Dee males sampled were all in the stage I tube condition. Running ripe testes were first encountered on 20 September and many were in stages IV and V, but stage I+ was still represented. During October running ripe stages were more prevalent, but the majority were in stages III and IV, with V fairly abundant. The only sample taken in November was caught in the Alwen and was composed almost entirely of running males. Some of these running ripe testes taken from the Alwen during November (the general spawning period of the Dee) were slightly smaller than the stage VI, and there was little doubt that these had already shed some sperm. A very interesting sample of males was taken in the race leading from the salmon keeping tanks; it was composed entirely of ripe males (King, Jones and Orton 1939).

By 20 December the testes showed definite signs of spawning and were collapsing to sizes similar to those of stages III and II. Several different spending stages were recorded, ranging from the fullish VII–III+'s to the much collapsed VII–I+'s. All the latter contained sperm either in large white patches or relict spots. Sperm was present with one exception in all ducts. Stages VII–I+ were slightly more numerous in the January samples, but sperm was still present; stages VII–III and VII–II+ were common and many still running on pressure. During February testes in stages VII–I+ were predominant, many had empty ducts, and others patches or spots of relict sperm only. Empty testes were still more prevalent during March, yet many showed relict sperm and one stage

VII–III was recorded from the Alwen. During April, the fawn coloured testes were common and the peachy types were devoid of sperm.

The cycle of the testis as shown by the Dee (1938–9 inter-smolt) samples may be summarized as follows:

July	Developing, mainly I+, some II's, II+'s and III's.
August	Developing, some III's, and IV's quite commonly seen.
September	Developing, IV's more frequent; one running (20 September).
October	Developing, V's and VI's dominant.
November	Spawning.
December	Spending and spent VII–III's, VII–II's and VII–I+'s.
January	Spent, more VII–I+'s.
February	VII–I+'s predominant (peachy, some without sperm).
March	Many without sperm, few with.
April	Fawny, no sperm.

During May 1939 the samples examined contained spent males and stage I tubes only; but during June some interesting hitherto unrecorded types of testes were noticed. These were of a greasy pinkish colour, the pinkiness being more diffuse and different from the tint of a peachy testis. Their ducts were large and wide and very reminiscent of the ducts of spent testes. These were probably recently spent testes developing for a second time. Their microscopic characters are described below. It is interesting to note that their development as shown by size was very much in advance of the normal developing testes. Other testes in a June 1939 sample were definitely developing, as shown by their increased width anteriorly. The further development and spending cycle was closely similar to that noticed above during the 1938–9 cycle.

Of all other rivers sampled only the Wnion, Dolgelley, N. Wales, can be treated in any detail. During June 1938 testes which may have been recovering spents were recorded. In July, the testes sampled were mainly in stage I+ with some in stage II, and towards the end of the month some in stage III were present. A month later testes in stage IV were recorded and became common during October. The first running ripe testis was taken on 9 November, and by 18 November several testes showed signs of spending. Stages VII–III and VII–II were plentiful in December. During February 1939 VII–II's changing into VII–I+'s were noticed. During March these predominated, but their ducts were generally full of sperm. A change over from a peachy to fawny stage VII–I+ was seen in April, one fish possessing one peachy and one fawny testis.

Developing testes (I+) were present in a sample recorded on 24 May, and on 5 June a large netted sample contained forty-four males of which thirty-five were in stage I showing no sign of developing, six were in stage I +, two in stage II and one in stage II +. Towards the end of June some probable recovering spents were evident.

3. HISTOLOGIC AND SPERMATOGENIC CHANGES

(a) *Stage I* (see figure 1, plate 20)

A large number of the thread-like stages have been sectioned. There is little variation in their histological structure. The outer tunica is thin and the crypts outlined only by occasional minute blood capillaries. Empty spaces are rare or absent, the crypts are poorly defined and apparently filled by large cells which are the resting germ cells (see figure 1). The resting germ cells have large clearly stained nuclei with small masses of darkly staining chromatin and one or two plasmosomes. The cytoplasm is unstained and the cell wall poorly defined (see also figures 2–4, plate 20). Migrating germ cells (figures 1 and 2, plate 20) are present in the earliest stages sectioned. These cells have smaller nuclei than the resting germ cells and are not always spherical in shape. Various stages are present, the youngest being most numerous around the periphery of the testes. Their inward migration seems to be along definite paths which are defined by the walls of the crypts (and blood capillaries). As they migrate inwards the germ cells increase in size and their nuclei become more spherical in outline and take on a lighter stain. When they enter the future crypts their growth is very apparent. The plasmosome becomes more pronounced and is situated, if unpaired, slightly acentral. Chromatin masses appear around the periphery of the nucleus, though the passage of these into the cytoplasm (as noticed by Turner 1919) has not been recorded. The largest resting germ cell is about three to four times the size of the smallest migrating forms as seen near the periphery of the testes.

No cells other than those resting and migrating germ cells and rare connective tissue cells are present in the stage I testis. The resting germ cells with their lightly staining homogeneous cytoplasm completely fill the region which is later to be the crypt space. The germ cells are not grouped so clearly as in the perch (Turner 1919), and they increase in number by addition from the migrating germ cells. It is very noticeable that, as the resting germ cells increase in number, there is a corresponding decrease in the numbers of the migrating germ cells (see figures 1–3, plate 20). Various stages in the populating of the crypts by these cells have been seen, though no mitotic figures have been noticed. Similar germ cells have been described by Foley (1926), Turner (1919), Matthews (1938).

The blood supply of the stage I testis is well developed, a large blood vessel being present along the inner lateral periphery. Blood corpuscles may be seen in the weakly differentiated crypt walls. No further changes occur in the stage I testes.

(*b*) *Stages I+ to II*

An increase in size of the testis is correlated with the appearance of spermatogonia. The germ cells give rise to the spermatogonia; the transformation may be summarized as:

(i) A decrease in the size of the nucleus,
(ii) The cells become more darkly staining.

Their appearance is closely related to size, the wider testes of stages I+ and II having the greater number of spermatogonia. Figures 2 and 3, plate 20, show an early developing testis where the groups of spermatogonia are localized. Figure 2, plate 20, shows clearly the larger germ cell nuclei, the darkly staining resting spermatogonia and the very apparent dividing spermatogonia. Except where spermatogonia are present the crypt regions are poorly defined. The spermatogonia appear indiscriminately in the testis; there is no indication that they first tend to appear near the periphery. Migrating germ cells can still be seen in a I+ testis.

As in perch (Turner 1919) each spermatogonium divides to give rise to a group of descendants which are enclosed in a "cyst" and undergo all subsequent divisions at the same time. The secondary spermatogonia appear early, before all of the germ cells have given rise to primary spermatogonia (figure 4, plate 20). Some were noticed in a testis where most of the cells were resting germ cells (figure 3, plate 20).

Figures 4 and 5, plates 20, 21, show sections of testes in stage II. Here the spermatogenesis is well advanced, but resting germ cells are still present. Primary and the smaller secondary spermatogonia are apparent, and the spermatocytes make their appearance. The latter are smaller than the secondary spermatogonia, there being no increase in size of the primary spermatocyte as in Dipnoans (Swingle 1921). The spermatocytes are spherical darkly staining cells with a rather peculiar uneven distribution of the nuclear chromatin, which gives them the appearance of being mottled. (This is not clearly shown in the photographs.) Figure 7, plate 21, shows smaller magnification of the testes of figures 4 and 5, plates 20, 21. Note the appearance of the spermatocytes in groups as mentioned above.

(c) *Stages III to V*

As noticed macroscopically, there is a pronounced change in the colour of the testes when developing from stages II to III. The change to white is correlated with the appearance of sperm in appreciable quantity. Sperm are produced very shortly after the appearance of spermatocytes and spermatids. It was found very hard to differentiate between sperm and spermatid in the Ehrlich's haematoxylin preparations. They are very similar, spermatids being only slightly more lightly stained than the sperm. Both cells are of a similar shape, the sperm being rounded at the head and possibly very slightly smaller than the spermatid. Figure 8, plate 21, shows a testis in stage III. The crypts contain much sperm, but the intercrypt regions are quite pronounced. Here may be seen spermatocytes and spermatids.

Further development of the testes consists mainly of an increase in size related to the formation of more sperm. The crypts swollen with sperm occupy the main mass of the testes and the crypt walls are greatly attenuated, as is seen clearly in figure 9, plate 21 (stage V–VI).

(d) *Spending and spent testes*

Following the extrusion of sperm, the testes decrease in size and the crypts become empty. The most pronounced change occurs in the structure of the walls of the crypt (stroma) and the tunica of the testis. The general appearance of the section denotes a collapse. This is well shown in figure 10, plate 22. The crypt walls have the appearance of contracted elastic tissue and now show as relatively thick bands forming an open meshwork containing more or less sperm, and the outer tunica is considerably thicker.

A further stage in this collapse and emptying is shown in figure 11, plate 22. This microscope section is from a stage VII–II gonad which had sperm in the duct and spots of relict sperm in the collapsed peachy testicular portion.

Eventually the testicular portion is completely emptied of sperm (figures 11, 12, plate 22). In figure 11, plate 22, is seen a transverse section of the collapsed testis, the still enlarged "duct" and very thick outer tunica. The testicular duct is a channel running along the edge of the testis, and into this channel all sperm leaving the testis passes until it eventually reaches the true duct in the narrow posterior portion of the testis which conveys the sperm to the cloaca. The outer tunica in the spent gonad becomes thicker owing to contraction on emptying and is thrown into longitudinal ridges (see figures 11 and 12, plate 22). The number of blood capillaries in the crypt walls of a stage VII–I + testis is great. It is quite possible that these may supply the elements for the re-absorption of the redundant sperm.

The fawny type of testis, VII–I +, which was the macroscopic end-stage of spawning, is very typical, showing a large empty testicular duct region (above the large blood vessel) and the evacuated crypts, some of which contain large connective tissue cells, some being empty and others already containing an appreciable number of resting germ cells. This stage still retains the thick longitudinally ridged outer tunica. Such testes are well on the way to recovering, and as a small percentage was taken after the end of the smolt migration amongst the parr it is quite possible that these testes will redevelop and that another spawning may occur during the following November. During June and early July some large well-developed testes were taken. These were a pinky whitish colour, the pinkiness being diffuse, and as these were well advanced in development it was thought that they may be recovering spents developing for the second time. This conclusion was also strengthened by the condition of their ducts which were relatively large in size and reminiscent of the ducts of the stage VII–II and VII–I + testes. Microscopically they do not differ from normal stage II and II + testes. In some the tunica was slightly thicker than normal.

4. The stage I tubes of smolts

It was suspected, that of the large number of smolt gonads in the stage I tube condition, some may show traces of a previous spawning. Consequently, a large number of these gonads was sectioned and examined. Most of these were normal stage I tubes showing the very characteristic general structure and the typical migrating and resting germ cells. A small number, however, had a thickish tunica in the region of the duct, reminiscent of the spent gonad. No definite conclusions can be drawn about this type until more is known about the spent condition following the VII–I fawny stage.

5. Summary

The histological changes in the seasonal sexual cycle of the male salmon parr have been described, the nomenclature recommended by the International Council being used for the various arbitrary macroscopic stages.

The undeveloped tube-like stage I gonad showed no spermatogenic changes other than the migration and growth of typical germ cells. This type of testis was taken throughout the year. Testes showing slight

widening anteriorly, stage (I+), show the presence of spermatogonia. Further growth and development of the testes are related to the spermatogenic changes which result in the production of some sperm in late stage II and an abundance in stages III, IV and V. In stages IV and V the increase in the size of the testis is related to the increase in abundance of sperm. These changes begin in June–July and ripe sperm is present during October–November.

The changes brought about by the general collapse of the testes after spawning, and the accompanying structural modifications, have been described.

Some testes which may be developing to ripen for a second time have been examined.

The tube-like stage I testes from smolts were found to be mostly, if not entirely, immature, and similar to those of parr.

No ripening or ripe testes were found in male smolts.

The author wishes to offer his sincere thanks to Professor J. H. Orton for valuable criticism and assistance; to the Dee Fishery Board, especially Mr King and the bailiffs; to the Dovey, Mawddach and Glaslyn Fishery Board and their clerk Mr Pugh; to all other Fishery Boards who so kindly forwarded samples, and to Mr R. A. Fleming who took the photomicrographs.

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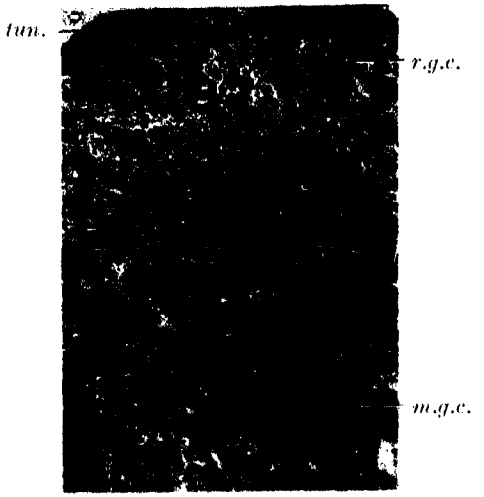

FIGURE 1

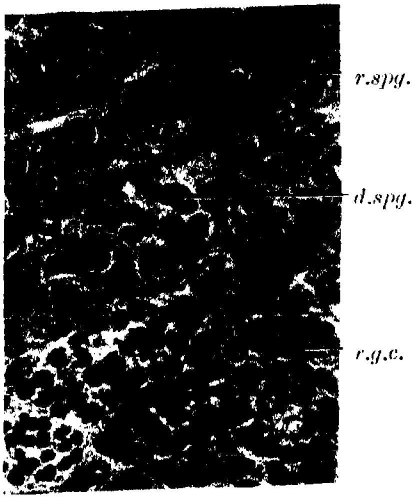

FIGURE 3

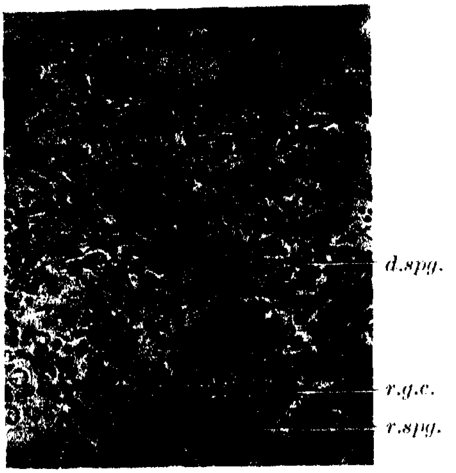

FIGURE 2

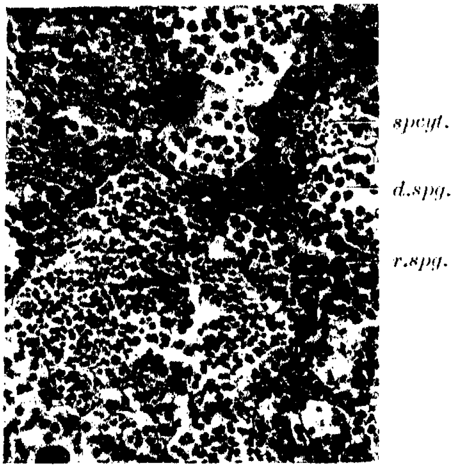

FIGURE 4

Early developmental stages of testis.

FIGURE 1. Stage I tube, × 245, showing resting and migrating germ cells, thin tunica and indications of crypt walls as stroma.

FIGURE 2. Stage I +, × 245, showing dividing spermatogonia.

FIGURE 3. Stage I +, × 525, clusters of dividing spermatogonia and few resting spermatogonia.

FIGURE 4. Stage II, × 245. First appearance of spermatocytes. Note groups of similar cells in 'cysts'.

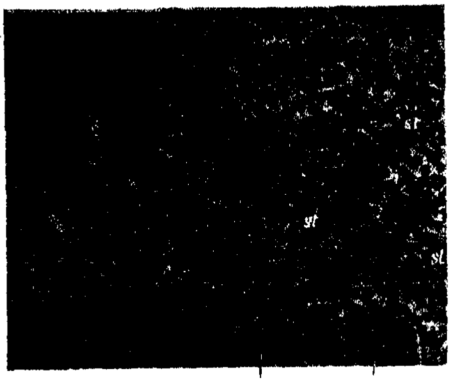

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

FIGURE 9

Later developmental stages.

FIGURE 5. Late stage II, × 245. Fewer of the younger cells and stronger indications of crypt walls as stroma.

FIGURE 6. Stage II, × 525. Spermatogonia and spermatocytes; note rare resting germ cells.

FIGURE 7. Stage II, × 52·5. Low power photomicrograph of figure 5; note absence of definite crypt walls.

FIGURE 8. Stage III, × 245. Abundance of sperm, bounded by younger cells.

FIGURE 9. Stage VI, × 245. Ripe testis with abundance of sperm and very tenuous crypt walls.

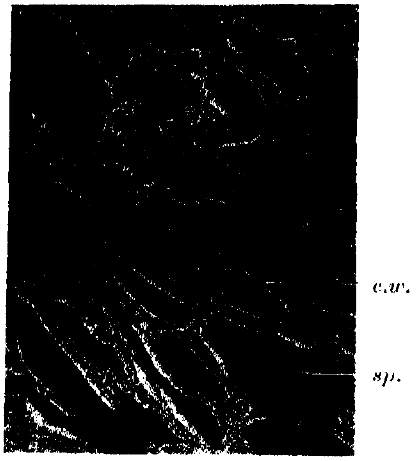

FIGURE 10

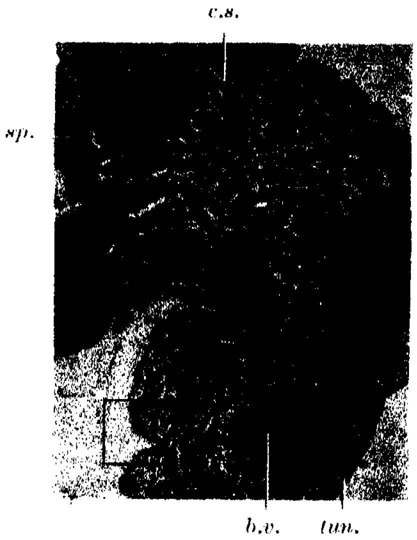

FIGURE 11

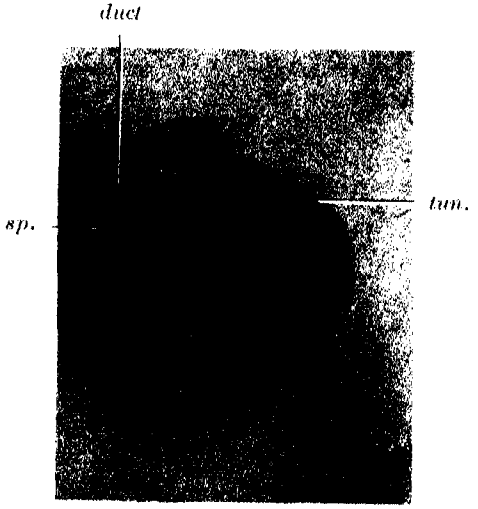

FIGURE 12

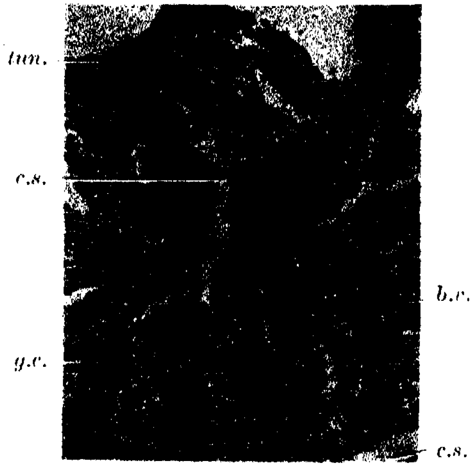

FIGURE 13

Spending and spent testes.

FIGURE 10. Stage VII–III, ×245, showing effect of the general collapse after spawning and thick crypt walls.

FIGURE 11. Stage VII–III, ×52·5. Collapsed testis with sperm in duct only. Note the thick folded outer tunica.

FIGURE 12. ×19. Low power of figure 11. Large duct full of sperm is shown.

FIGURE 13. ×245. Enlargement of portion of figure 11 bounded by square. Note thick tunica and numerous blood corpuscles and resting germ cells, the latter suggestive of a re-development.

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Abbreviations used on plates

b.v.	blood vessel.	*m.g.c.*	migrating germ cells.
c.s.	crypt space.	*r.g.c.*	resting germ cells.
c.w.	crypt wall.	*r.spg.*	resting spermatogonia.
d.spg.	dividing spermatogonia.	*spcyt.*	spermatocytes.
du.	duct.	*sp.*	sperm.
g.c.	germ cells.	*tun.*	tunica.

Structural analysis of *Oenothera* complexes

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In *Oenothera* species each functional gamete normally contains seven chromosomes, which together may be described as a gametic complex. Each species has two such complexes, either identical or different from one another. The differences are (1) *structural*, the chromosomes of two complexes differing in one or more interchanges, and (2) *genic*, the complexes differing in gene content. The present account deals principally with the first kind of difference. A complete and accurate analysis is a prerequisite for various kinds of genetic investigation of *Oenothera*.

The seven chromosomes of each gametic complex are composed of fourteen end-pairing segments, no two of which are alike, and of seven middle pieces probably including the centromeres. The middle parts of the chromosomes have so far largely defied analysis. However, it is possible to specify and define each of the end-pieces, and therefore each chromosome in terms of them, on physiological grounds, namely, their property of pairing at meiosis. Owing to the interchanges by which complexes differ, different metaphase associations of chromosomes are produced at meiosis by the pairing of similar chromosome ends when different complexes are combined. Considerable advances in such cytological analysis of many complexes have been made, especially by Emerson and Sturtevant (1931, 1932), Emerson (1936) and Cleland (1935, 1937). Renner (1933, 1938) has made similar analyses by employing breeding methods in which use is made of marker genes to test whether particular chromosomes are linked in segregation.

It is almost inevitable that the definitions employed in these two systems of analysis should be at variance with one another. For it is unlikely that fourteen different entities, the end-pairing segments of the chromosomes, would be labelled in the same way in separate arbitrary schemes. But, happily, the schemes of the American authors and of Renner are not entirely independent. Unfortunately, however, the points of difference in the two systems of definition have not been recognized, with the result that Cleland (1937, p. 485) concluded that the only formula for *rigens* common to his four possibles and Renner's two must be the correct one. But, as the two sets of possibilities were inferred on two distinctly different systems of definition, i.e. labelling of chromosome ends, it is mere chance that there was even one in common. Further computations, e.g. those of Renner (1938), based on this inference are therefore vitiated.

The present author has been accumulating data with a view to fitting the complexes described by Gates and Catcheside (1932) into the system outlined by Emerson and Sturtevant (1931). The new data have served to bridge the gap between Renner's work and that of the American authors.

In the analysis outlined below only those items in the new hitherto unpublished data or in the published data which contribute to the analysis are listed. There are, of course, many additional determinations of the chromosome configuration in other combinations of the complexes here discussed. They do not, however, promote the analysis, though they fit and confirm the conclusions in every test that can be applied. On the other hand, it is impossible to point to any datum that definitely disagrees with the conclusions reached. In fact, on several occasions during the work a prediction was made, and later verified by further experiment, that an apparent misfit was in reality an error of observation in one respect or another. Any hitherto unpublished cytological determination is referred to as "C. unpubl." in the text under the complex where the datum is used. In some cases it will be found that the chromosome configuration is different from that listed in the compendium of Gates and Ford (1938); the present record is a correction of the previous one.

An extension and modification of the definitions proposed by Emerson and Sturtevant (1931) has been made. The reasons for the modifications are set forth in the places where they are made. The alterations and extensions have been made with a view to the least possible disturbance of previously published analyses.

The full arguments and computations have not been set out in detail at places where they would add unnecessarily to the length and tedium of the paper. Enough information has been given to point out the method used in the selection or elimination of different possibilities at such places.

ALTERATIONS TO DEFINITION OF CHROMOSOME PAIRING ENDS

Emerson and Sturtevant (1931) proposed the following definitions of chromosome end-pairing segments for a number of *Oenothera* complexes:

ʰ*Hookeri*	1.2	3.4	5.6	7.8	9.10	11.12	13.14
flavens	1.4	2.3	5.6	7.8	9.10	11.12	13.14
velans	1.2	3.4	5.8	6.7	9.10	11.12	13.14
gaudens	1.2	5.6					
ʰ*franciscana*	1.2	3.4	5.6	7.10	8.9	11.12	13.14
"*N*"	1.2	3.4	5.6	7.14	8.13	9.10	11.12
jugens	1.4	7.14	8.9				

These definitions served to define all the chromosome ends, except 11 and 12 which are still equivalent. No two of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13 and 14 are now equivalent. Unfortunately, the original strain of "*N*" was lost, and the impossibility of testing new complexes with it (and, indeed, checking the determinations with old ones) has inevitably led to difficulties. It seems best to cut the Gordian knot by discarding the definitions sealed by "*N*" and *jugens*. In doing so, it must be understood clearly that retaining the chromosome definitions based upon ʰ*Hookeri*, *flavens*, *velans*, *gaudens* and ʰ*franciscana*, we have that:

1 is equivalent to 2
3 ,, 4
5 ,, 6
7 ,, 8
9 ,, 10
11.12 ,, 13.14

^{h}Blandina (an alethal complex of *O. Lamarckiana*).

^{h}*blandina*.^{h}*Hookeri*	6, 2, 2, 2, 2	Cleland 1937; Catcheside 1939*a*.
^{h}*blandina*.*flavens*	6, 4, 2, 2	Cleland 1937; Catcheside 1939*a*.
^{h}*blandina*.*velans*	8, 2, 2, 2	Cleland 1937; Catcheside 1937.
^{h}*blandina*.*excellens*	4, 2, 2, 2, 2, 2	Cleland 1937; C. unpub.

This complex has two ^{h}*Hookeri*, not *flavens*, chromosomes, namely, 1.2 and 3.4; and one ^{h}*Hookeri*, not *velans*, chromosome, namely, 5.6 or 7.8. Now *excellens* has a chromosome structure identical with that of ^{h}*franciscana* in respect of the arrangement of the end-segments. ^{h}*Blandina* has one *excellens*, not ^{h}*Hookeri*, chromosome, namely, 7.10 or 8.9; both of these are incompatible with 7.8 and therefore ^{h}*blandina* must have 5.6. The fourth chromosome like ^{h}*Hookeri* must be 11.12 or 13.14.

We know that 7.10 and 8.9 are equivalent, as also are 11.12 and 13.14. We may select either one of each pair, say 7.10 like *excellens* and 11.12 like ^{h}*Hookeri*. This fixes the definition of 7, 8, 9 and 10, no two of which are now equivalent; it also defines the difference between 11.12 and 13.14 but leaves 11 equivalent to 12 and 13 equivalent to 14. In ^{h}*blandina* there is an interchange between 8.9 and 13.14, and since 13 is still equivalent to 14 it does not matter which of the two possibilities we select. If our selection is 8.13 + 9.14 we have that

^{h}*blandina* is 1.2 3.4 5.6 7.10 11.12 8.13 9.14

and that 13 and 14 are now fixed.

Catcheside (1939*a*) has shown that two X-ray induced interchanges of ^{h}*blandina* have the following structures:

$$\textit{blandina}\text{-A is}\quad 1.2\quad 5.6\quad 7.10\quad 8.13\quad 9.14\left\langle\begin{array}{lll}3.11 & 4.12 & (a)\\ 3.12 & 4.11 & (b)\end{array}\right.$$

blandina-B is 1.4 2.3 5.6 7.10 11.12 8·13 9.14

Latifrons (the alethal complex of Shull's *O. rubricalyx*) is known (Emerson and Sturtevant 1931) to have 1.2, 3.4, 5.6, (7.10 or 8.9) and (11.12 or 13.14):

latifrons.^{h}*blandina* 4, 2, 2, 2, 2, 2 C. unpub.

Clearly *latifrons* has two more chromosomes like ^{h}*blandina*, and these can only be 7.10 and 11.12. For, if *latifrons* had 8.13 and 9.14 like ^{h}*blandina* it would need to have 7.10 and 11.12 like ^{h}*franciscana*; this arrangement would give seven pairs with ^{h}*blandina*.

Latifrons is 1.2 3.4 5.6 7.10 11.12 8.14 9.13

^{h}Purpurata (the complex of the homozygous species *O. purpurata* from northern Germany).

^{h}*purpurata*.^{h}*Hookeri*	4, 2, 2, 2, 2, 2	C. unpub.
^{h}*purpurata*.*flavens*	4, 4, 2, 2, 2	C. unpub.
^{h}*purpurata*.*velans*	6, 2, 2, 2, 2	C. unpub.
^{h}*purpurata*.^{h}*blandina*	4, 2, 2, 2, 2, 2	Gates and Catcheside 1931.
^{h}*purpurata*.*latifrons*	6, 2, 2, 2, 2	C. unpub.

This complex has two more chromosomes like* ^{h}*Hookeri* than it has like *flavens*; these are 1.2 and 3.4. It also has one more like ^{h}*Hookeri* than like *velans*, viz. 5.6 or 7.8. Chromosome 5.6 must be this one, for if ^{h}*purpurata* had 7.8 and not 5.6 it must also have two of the three chromosomes (7.10, 8.13 and 9.14) by which ^{h}*blandina* differs from ^{h}*Hookeri*; obviously that is impossible.

Further, ^{h}*purpurata* has two other chromosomes like ^{h}*Hookeri*, i.e. two of 9.10, 11.12 and 13.14, and two others like ^{h}*blandina*, i.e. two of 7.10, 11.12, 8.13 and 9.14. These requirements can be satisfied only if ^{h}*purpurata* has 11.12. But it also has two more chromosomes like *latifrons*, i.e. two of 7.10, 11.12, 8.14 and 9.13; 11.12 is one of these, and the only possibility for the second one is 7.10. The remaining chromosomes of ^{h}*purpurata* are 8.9 and 13.14.

^{h}*Purpurata* is 1.2 3.4 5.6 7.10 8.9 11.12 13.14

It follows that ^{h}*purpurata* is equivalent to ^{h}*franciscana*, *excellens* and a number of Californian complexes (Cleland 1935).

s-Albicans (the egg complex of *O. suaveolens*, a European species; the similar complex of *O. biennis* will be considered later).

s-*albicans*.^{h}*Hookeri*	14	Cleland and Blakeslee 1930; C. unpub.
s-*albicans*.*flavens*	12, 2	Cleland 1928; C. unpub.
s-*albicans*.^{h}*purpurata*	12, 2	C. unpub.
s-*albicans*.^{h}*blandina*	14	C. unpub.

It has one *flavens*, not ^{h}*Hookeri*, chromosome, namely, 1.4 or 2.3. These two are still equivalent and we may select either, say 1.4. This fixes 1, 2, 3 and 4, no two of which are now equivalent. s-*Albicans* also has one

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* The phraseology is necessarily compressed to save circumlocution. A statement such as this must be taken to mean that the specified number of chromosomes in one complex have a structure in respect of their end-segments identical with that of the corresponding chromosomes in a second complex but different from that of those in a third complex. From such a fact we may deduce the structure of certain chromosomes in the first complex.

^{h}*purpurata*, not ^{h}*Hookeri*, chromosome, i.e. 7.10 or 8.9. But since it has no chromosome like ^{h}*blandina*, the one like ^{h}*purpurata* must be 8.9.

s-*Albicans* has 1.4 8.9.

Before proceeding further with the analysis it will be convenient to summarize the definitions that have been proposed, as follows:

Definitions

1.2: present in ^{h}*Hookeri* and *gaudens* but not in *flavens*.
1—in the pair of *flavens*.s-*albicans*.
2—in the ring of *flavens*.s-*albicans*.
3.4: present in ^{h}*Hookeri* but not in *gaudens* or *flavens*.
3—in the ring of *flavens*.s-*albicans*.
4—in the pair of *flavens*.s-*albicans*.
5.6: present in ^{h}*Hookeri* and *gaudens* but not in *velans*.
5—with 8 in *velans*.
6—with 7 in *velans*.
7.8: present in ^{h}*Hookeri* but not in *gaudens* or *velans*.
7—in a pair in ^{h}*franciscana*.^{h}*blandina*.
8—in the ring in ^{h}*franciscana*.^{h}*blandina*.
9.10: exchanged with 7.8 in ^{h}*franciscana*.
9—in the ring in ^{h}*franciscana*.^{h}*blandina*.
10—in a pair in ^{h}*franciscana*.^{h}*blandina*.
11.12: the remaining chromosome of ^{h}*Hookeri* when the other six have been specified.
11—not yet differentiated from 12.
12—not yet differentiated from 11.
13.14: exchanged with 8.9 in ^{h}*blandina*.
13—with 8 in ^{h}*blandina*.
14—with 9 in ^{h}*blandina*.

ANALYSIS OF VARIOUS COMPLEXES*

Rubricalyx-α (the alethal complex of Gates' *O. rubricalyx*).

rubricalyx-α.^{h}*Hookeri*	4, 2, 2, 2, 2, 2	C. unpub.
rubricalyx-α.*flavens*	4, 4, 2, 2, 2	C. unpub.

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* Since this manuscript was completed, S. M. Sikka has published a paper (*J. Genet.* **39**, 309, 1940) in which he discusses some of the complexes dealt with in this article. His analyses depend upon the definitions of the chromosome end-segments described in the previous section and his conclusions agree with those described in this section for ^{h}*purpurata* and *rubricalyx*-α, but not for *rigens*.

rubricalyx-α.velans	6, 2, 2, 2, 2	C. 1937.
rubricalyx-α.gaudens	10, 2, 2	C. unpub.
*rubricalyx-α.*ʰ*blandina*	4, 2, 2, 2, 2, 2	Gates and Catcheside 1932; C. unpub.
*rubricalyx-α.*ʰ*purpurata*	6, 2, 2, 2, 2	Gates and Catcheside 1932; C. unpub.

There are two chromosomes like ʰ*Hookeri* and not like *flavens*; therefore *rubricalyx-α* has 1.2 and 3.4. It has one chromosome like ʰ*Hookeri* and not like *velans*, namely, 5.6 or 7.8. One pair with *gaudens* is given by 1.2. If *rubricalyx-α* has 7.8, and knowing that it must also have two of 9.10, 11.12 and 13.14, with the third of these interchanged with 5.6, there could be no remaining possibility for a second pair with *gaudens*. Hence *rubricalyx-α* has 5.6 and 7.8 is exchanged with one of 9.10, 11.12 and 13.14. Also, *rubricalyx-α* has two more chromosomes like ʰ*blandina*, i.e. two of 7.10, 11.12, 8.13 and 9.14; it must therefore have 11.12 and either 7.10, 8.9 and 13.14 or 7.14, 8.13 and 9.10. But in *rubricalyx-α.* ʰ*purpurata* there is a ring of six chromosomes; therefore *rubricalyx-α* cannot have 7.10, 8.9 and 13.14.

Rubricalyx-α is 1.2 3.4 5.6 7.14 8.13 9.10 11.12.

In a like manner, it may be shown (cf. Catcheside 1939*b*) that ʰ*decipiens*, the alethal complex of *O. erythrina*, has a similar structure.

Rigens (the egg complex of *O. muricata*, a European species).

*rigens.*ʰ*Hookeri*	6, 2, 2, 2, 2	Cleland 1932.
rigens.flavens	6, 4, 2, 2	Cleland 1932.
rigens.velans	8, 2, 2, 2	Cleland 1933.
*rigens.*ʰ*purpurata*	8, 2, 2, 2	C. unpub.
rigens.gaudens	6, 2, 2, 2, 2	C. unpub.
rigens.rubricalyx-α	6, 2, 2, 2, 2	C. unpub.
*rigens.*ʰ*blandina*	8, 2, 2, 2	Cleland 1937; C. unpub.

Rigens has two more chromosomes like ʰ*Hookeri* than *flavens*; therefore it has 1.2 and 3.4. It has one more chromosome like ʰ*Hookeri* than *velans*, namely, 5.6 or 7.8. Lastly, there is one more chromosome like ʰ*Hookeri* than ʰ*purpurata*, namely, 7.8 or 9.10. Therefore, *rigens* has 5.6 and 9.10 or 7.8 alone. With *gaudens*, one of the four pairs is given by 1.2; unless one of the other three is given by 5.6 it would be impossible to have four chromosomes in common between *rigens* and ʰ*Hookeri* and between *rigens* and *gaudens*. Hence *rigens* has 5.6 and 9.10. The three remaining chromosomes are compounded of the chromosome end-segments 7, 8, 11, 12, 13 and 14 taken two at a time; there are eight ways in which a ring of six chromosomes with ʰ*Hookeri* could be secured from these, viz.

7.11	8.14	12.13
	8.13	12.14
7.12	8.14	11.13
	8.13	11.14
7.13	8.11	12.14
	8.12	11.14
7.14	8.11	12.13
	8.12	11.13

But *rigens* cannot have 7.14 or 8.13 like *rubricalyx-α* or there would be more than four pairs in their combination. The four arrangements with 7.14 or 8.13 may be excluded in other ways: (1) Since *gaudens.rigens* has four pairs, *gaudens* must have two chromosomes like those of *rigens*, made up from 7, 8, 11, 12, 13 and 14. But *gaudens* cannot have 7.14 or 8.13, for *gaudens.rubricalyx-α* has a ring of ten chromosomes and two pairs (C. unpub.), the latter being given by 1.2 and 5.6. Hence for the cases where *rigens* has 7.14 or 8.13, *gaudens* would be required to have 7.11 and 12.14, 7.12 and 11.14, 8.11 and 12.13, or 8.12 and 11.13 respectively. In each case, therefore, *gaudens.rubricalyx-α* would have a ring of four chromosomes, which is inconsistent with observation. (2) *Rigens*.ʰ*blandina* has a ring of eight and three pairs, the latter being accounted for by 1.2, 3.4 and 5.6. Therefore, *rigens* cannot have 8.13. Nor can it have 7.14, for in such a case it would also have 8.11 and 12.13 or 8.12 and 11.13, either of which would give a ring of four chromosomes with 8.13 and 11.12 of ʰ*blandina*. Therefore the four *rigens* possibilities having 7.14 or 8.13 are excluded.

Rigens is	1.2	3.4	5.6	9.10	7.11	8.14	12.13	(*a*)
					7.12	8.14	11.13	(*b*)
					7.13	8.11	12.14	(*c*)
					7.13	8.12	11.14	(*d*)

These four possibilities appear to be the same as those inferred by Cleland (1937, pp. 484–485), but since they have been derived on independent systems it does not follow that the possibilities are identical each to each.

Gaudens (a complex of *O. Lamarckiana*, a European species) is known to have 1.2 and 5.6 (Emerson and Sturtevant 1931).

gaudens.latifrons	8, 2, 2, 2	Emerson 1930.
gaudens.rigens	6, 2, 2, 2, 2	C. unpub.
gaudens.blandina-A	8, 2, 2, 2	Catcheside 1939*a*.
gaudens.^{h}*blandina*	10, 2, 2	Cleland 1937; Catcheside 1937.
gaudens.^{h}*franciscana*	10, 2, 2	Emerson and Sturtevant 1931.
gaudens.^{h}*Hookeri*	10, 2, 2	Cleland and Blakeslee 1930.

Gaudens has one more chromosome like *blandina*-A than like ^{h}*blandina*, i.e. one of 3.11, 3.12, 4.11 and 4.12. It also has one more chromosome like *latifrons* than like ^{h}*Hookeri*, ^{h}*franciscana*, ^{h}*blandina* or *rubricalyx*-α, i.e. 8.14 or 9.13. Further, it has two more chromosomes like *rigens* than like ^{h}*Hookeri*. These must be two of the three *rigens* chromosomes that give the ring of six with ^{h}*Hookeri*. There are three ways of selecting these two chromosomes from each of the four *rigens* possibilities, thus making twelve different selections:

Like *rigens* (*a*)	Like *rigens* (*b*)	Like *rigens* (*c*)	Like *rigens* (*d*)
7.11 + 8.14	7.12 + 8.14	7.13 + 8.11	7.13 + 8.12
7.11 + 12.13	7.12 + 11.13	7.13 + 12.14	7.13 + 11.14
8.14 + 12.13	8.14 + 11.13	8.11 + 12.14	8.12 + 11.14

Two, viz. 8.11 + 12.14 and 8.12 + 11.14, would give a ring of four chromosomes with *latifrons* 8.14 + 11.12 and are therefore impossible. Four more, viz. 7.13 + 8.11, 7.13 + 12.14, 7.13 + 8.12 and 7.13 + 11.14, are impossible, as they would not allow the third chromosome like *latifrons*, namely, 8.14 or 9.13. This means that *rigens* (*c*) and (*d*) possibilities must be discarded, as neither of them is compatible with other *gaudens* requirements.

Consequently, *rigens* has 1.2, 3.4, 5.6, 9.10, 8.14 and either 7.11 + 12.13 or 7.12 + 11.13. But ends 11 and 12 are still equivalent and we can define them by selecting one of the two alternative formulae, say 7.12 + 11.13.

Rigens is 1.2 3.4 5.6 9.10 8.14 7.12 11.13

and the definitions of 11 and 12 are

11—with 13 in *rigens*,
12—with 7 in *rigens*.

This reduction in the number of the *rigens* formulae leaves only three possibilities for the two further chromosomes like *gaudens*. Of them, one, namely, 7.11 + 12.13, is impossible as it would not permit a third chromosome like *blandina*-A, i.e. one of 3.11, 3.12, 4.11 and 4.12, since both the ends 11 and 12 would be used up. Therefore *gaudens* has 8.14 like *latifrons* and *rigens* and either 7.12 or 11.13 like *rigens*. The possible formulae for *gaudens* are:

1.2	5.6	8.14	7.12	3.11	4.9	10.13	(i)
					4.10	9.13	(ii)
					4.13	9.10	(iii)
				4.11	3.9	10.13	(iv)
					3.10	9.13	(v)
					3.13	9.10	(vi)
			11.13	3.12	4.9	7.10	(vii)
					4.10	7.9	(viii)
					4.7	9.10	(ix)
				4.12	3.9	7.10	(x)
					3.10	7.9	(xi)
					3.7	9.10	(xii)

Many of these possibilities may be eliminated in the following ways:

(1) *Gaudens* cannot have 9.13 like *latifrons* as well as 8.14; hence (ii) and (v) are impossible.

(2) *Gaudens* cannot have 9.10 like ʰ*Hookeri*; formulae (iii), (vi), (ix) and (xii) are therefore impossible.

(3) *Gaudens* cannot have 7.10 like ʰ*franciscana*; therefore formulae (vii) and (x) are impossible.

Gaudens is one of	1.2	5.6	8.14	7.12	10.13	3.11	4.9	(*a*)
						4.11	3.9	(*b*)
				7.9	11.13	3.12	4.10	(*c*)
						4.12	3.10	(*d*)

Flectens (the pollen complex of Renner's strain of *O. cruciata*, derived from Lake George, N.Y., U.S.A. *fide* Cleland 1937).

flectens.ʰ*Hookeri*	6, 4, 4	Renner 1938.
flectens.*flavens*	6, 4, 2, 2	Renner 1933, 1938.
flectens.*velans*	6, 4, 4	Renner 1933, 1938.
flectens.*rigens*	8, 4, 2	Renner 1933, 1938.
flectens.*gaudens*	14	Renner 1938.

There are two chromosomes like *flavens*, but not like ʰ*Hookeri*; therefore *flectens* has 1.4 and 2.3. There is one *rigens*, not ʰ*Hookeri*, chromosome, i.e. one of 7.12, 8.14 and 11.13. But there is no pair in *gaudens*, therefore 8.14 is impossible. The chromosome like *rigens* must be 7.12 or 11.13.

The chromosomes 5.6 and 7.8 of *flavens* and ^{h}*Hookeri* and 5.8 and 6.7 of *velans* are either (1) in a ring of four chromosomes with *flectens* which must therefore have 5.7 and 6.8; or (2) in the ring of six chromosomes with *flectens* which must therefore have an arrangement of 5, 6, 7 and 8 with one of 9.10, 11.12 and 13.14.

On alternative (1) four chromosomes of *flectens* would be 1.4, 2.3, 5.7 and 6.8. A fifth, for the pair with *rigens*, must be 11.13. The remaining two are 9.11 and 10.14 or 9.14 and 10.11. Thus two possible *flectens* formulae are:

$$1.4\quad 2.3\quad 5.7\quad 6.8\quad 11.13 \left\langle \begin{array}{lll} 9.12 & 10.14 & \text{(i)} \\ 9.14 & 10.12 & \text{(ii)} \end{array}\right.$$

On alternative (2) the following formulae are possible, remembering that *flectens* must have 7.12 or 11.13 like *rigens*:

$$1.4\quad 2.3\quad \left[\begin{array}{l} 11.13\quad 12.14\quad \left[\begin{array}{l} 6.8\left\langle\begin{array}{lll}5.9 & 7.10 & \text{(iii)}\\ 5.10 & 7.9 & \text{(iv)}\end{array}\right. \\ 5.7\left\langle\begin{array}{lll}6.9 & 8.10 & \text{(v)}\\ 6.10 & 8.9 & \text{(vi)}\end{array}\right.\end{array}\right. \\ 7.12\quad 5.11\quad 6.8\left\langle\begin{array}{lll}9.13 & 10.14 & \text{(vii)}\\ 9.14 & 10.13 & \text{(viii)}\end{array}\right.\end{array}\right.$$

s-Albicans is known (p. 514 above) to have 1.4 and 8.9:

s-*albicans*.*flectens* 6, 2, 2, 2, 2 Renner 1938.

One of the pairs with *flectens* is given by 1.4; 2.3 of *flectens* must be in the ring of six chromosomes in s-*albicans*.*flectens*. We can write down the formulae for s-*albicans* that are compatible with each of the eight possibles for *flectens*. Each may then be tested in turn for their fit with other complexes.

With *flectens* (i):

$$1.4\quad 8.9\quad 5.7\quad 11.13\quad 10.14\left\langle\begin{array}{lll}2.6 & 3.12 & (a)\\ 2.12 & 3.6 & (b)\end{array}\right.$$

Neither of these will give the correct configuration (a ring of eight and a ring of six) with any of the four *gaudens* formulae. Hence *flectens* (i) is impossible.

With *flectens* (ii):

$$1.4 \quad 8.9 \quad 5.7 \quad 11.13 \quad 10.12\left<\begin{array}{lll}2.6 & 3.14 & (a)\\ 2.14 & 3.6 & (b)\end{array}\right.$$

Only (*b*) will fit any of the *gaudens* formulae, namely, *gaudens* (*a*).

With *flectens* (iii):

$$1.4 \quad 8.9 \quad 7.10 \quad 12.14 \quad 11.13\left<\begin{array}{lll}2.6 & 3.5 & (a)\\ 2.5 & 3.6 & (b)\end{array}\right.$$

These are impossible, for s-*albicans* cannot have two chromosomes, 8.9 and 7.10, like ^{h}*purpurata*. *Flectens* (iii) is impossible.

With *flectens* (iv):

$$1.4 \quad 8.9 \quad 5.10 \quad 12.14 \quad 11.13\left<\begin{array}{lll}2.6 & 3.7 & (a)\\ 2.7 & 3.6 & (b)\end{array}\right.$$

These are impossible for they would give a ring of four chromosomes, 11.12–*12.14*–14.13–*13.11*, with ^{h}*Hookeri*. *Flectens* (iv) is impossible.

With *flectens* (v):

$$1.4 \quad 8.9 \quad 5.7 \quad 12.14 \quad 11.13\left<\begin{array}{lll}2.6 & 3.10 & (a)\\ 2.10 & 3.6 & (b)\end{array}\right.$$

These are impossible for they would give a ring of four chromosomes, 11.12–*12.14*–14.13–*13.11*, with ^{h}*Hookeri*. *Flectens* (v) is impossible.

With *flectens* (vi):

Two chromosomes like *flectens* (vi) are 1.4 and 8.9. The other two are one of the following pairs: 11.13+12.14, 11.13+5.7, 11.13+6.10, 12.14+5.7, 12.14+6.10, and 5.7+6.10. Two of these pairs are impossible; for, 5.7+6.10 would give a ring of four chromosomes with 5.6 and 7.10 of ^{h}*purpurata* and 11.13+12.14 would give a ring of four with 11.12 and 13.14 of ^{h}*Hookeri*:

$$(a) \quad 1.4 \quad 8.9 \quad 11.13 \quad 5.7 \left[\begin{array}{l}
2.6\left<\begin{array}{lll}3.12 & 10.14 & \text{(i)}\\ 3.14 & 10.12 & \text{(ii)}\end{array}\right.\\
2.10\left<\begin{array}{lll}3.12 & 6.14 & \text{(iii)}\\ 3.14 & 6.12 & \text{(iv)}\end{array}\right.\\
2.12\left<\begin{array}{lll}3.6 & 10.14 & \text{(v)}\\ 3.10 & 6.14 & \text{(vi)}\end{array}\right.\\
2.14\left<\begin{array}{lll}3.6 & 10.12 & \text{(vii)}\\ 3.10 & 6.12 & \text{(viii)}\end{array}\right.
\end{array}\right.$$

Only (iii) and (vii) will fit any *gaudens* possibility, namely, *gaudens* (*a*) and (*b*).

(*b*) 1.4 8.9 6.10 11.13	2.5	3.12	7.14	(i)
		3.14	7.12	(ii)
	2.7	3.12	5.14	(iii)
		3.14	5.12	(iv)
	2.12	3.5	7.14	(v)
		3.7	5.14	(vi)
	2.14	3.5	7.12	(vii)
		3.7	5.12	(viii)

Formulae (i), (iv), (v) and (viii) will fit either *gaudens* (*a*) or (*b*).

(*c*) 1.4 8.9 12.14 5.7	2.6	3.11	10.13	(i)
		3.13	10.11	(ii)
	2.10	3.11	6.13	(iii)
		3.13	6.11	(iv)
	2.11	3.6	10.13	(v)
		3.10	6.13	(vi)
	2.13	3.6	10.11	(vii)
		3.10	6.11	(viii)

Formulae (v) and (vii) will fit *gaudens* (*c*) and (*d*).

(*d*) 1.4 8.9 6.10 12.14	2.5	3.11	7.13	(i)
		3.13	7.11	(ii)
	2.7	3.11	5.13	(iii)
		3.13	5.11	(iv)
	2.11	3.5	7.13	(v)
		3.7	5.13	(vi)
	2.13	3.5	7.11	(vii)
		3.7	5.11	(viii)

All except (iii), (v) and (vii) will fit one or more of the four *gaudens* possibilities.

With *flectens* (vii):

1.4 8.9 5.11 7.12 10.14	2.6	3.13	(*a*)
	2.13	3.6	(*b*)

Neither of these fits any of the four *gaudens* possibilities. Hence *flectens* (vii) is impossible.

With *flectens* (viii):

1.4	8.9	5.11	7.12	10.13	2.6	3.14	(*a*)
					2.14	3.6	(*b*)

Formula (*a*) will fit *gaudens* (*c*) and (*d*).

The tests of the s-*albicans* formulae have resulted in the elimination of all but three of the eight possible *flectens* formulae. These may, for convenience, be relabelled with letters, as follows:

Flectens is one of	1.4	2.3	5.7	6.8	11.13	9.14	10.12	(*a*)
				6.10	8.9	11.13	12.14	(*b*)
			6.8	5.11	7.12	9.14	10.13	(*c*)

Flectens (*a*) and (*b*) fit *gaudens* (*a*) and (*b*); *flectens* (*c*) fits *gaudens* (*c*) and (*d*).

Also, there are fifteen formulae that remain as possibilities for s-*albicans* but two are duplicates one of the other; thus there are fourteen that are actually different. We may summarize them, with the *gaudens* and *flectens* that they fit, as follows:

								fits *gaudens*	fits *flectens*
1.4	8.9	5.7	11.13	10.12	2.14	3.6	(*a*)	(*a*)	(*a*), (*b*)
				2.10	3.12	6.14	(*b*)	(*b*)	(*b*)
			12.14	2.11	3.6	10.13	(*c*)	(*c*), (*d*)	(*b*)
				2.13	3.6	10.11	(*d*)	(*c*), (*d*)	(*b*)
		6.10	12.14	2.5	3.11	7.13	(*e*)	(*c*), (*d*)	(*b*)
					3.13	7.11	(*f*)	(*c*), (*d*)	(*b*)
				2.7	3.13	5.11	(*g*)	(*a*)	(*b*)
				2.11	3.7	5.13	(*h*)	(*c*), (*d*)	(*b*)
				2.13	3.7	5.11	(*j*)	(*b*), (*c*), (*d*)	(*b*)
			11.13	2.5	3.12	7.14	(*k*)	(*b*)	(*b*)
				2.7	3.14	5.12	(*l*)	(*b*)	(*b*)
				2.12	3.5	7.14	(*m*)	(*a*)	(*b*)
				2.14	3.7	5.12	(*n*)	(*a*)	(*b*)
		5.11	7.12	10.13	2.6	3.14	(*o*)	(*c*), (*d*)	(*c*)

Five of these, namely, (*c*), (*d*), (*e*), (*f*) and (*h*), may be eliminated as they do not fit *flectens* and *gaudens* formulae that are mutually fitted. This leaves us with nine possibilities for s-*albicans*.

Curvans (the pollen complex of *O. muricata*).

curvans.^{h}*Hookeri*	8, 6	Cleland 1932.
curvans.*flavens*	6, 6, 2	Renner 1933, 1938.

curvans.excellens	8, 4, 2	Cleland 1933.
curvans.^{h}*purpurata*	8, 4, 2	C. unpub.
curvans.velans	14	Renner 1938.
curvans.rigens	14	Cleland 1926.
*curvans.*s-*albicans*	6, 4, 2, 2	Cleland 1931.
curvans.flectens	6, 4, 2, 2	Renner 1933, 1938.
curvans.gaudens	14	Renner 1938.

Curvans has one chromosome like *flavens*, but not ^{h}*Hookeri*, namely, 1.4 or 2.3. It also has one like *excellens* (and ^{h}*purpurata*), but not ^{h}*Hookeri*, namely, 7.10 or 8.9. Comparison of the configurations given with ^{h}*Hookeri* and ^{h}*purpurata* respectively, shows that the 8.9 and 7.10 chromosome of ^{h}*purpurata* not possessed by *curvans* must be in the ring of four in *curvans*. ^{h}*purpurata* with one of 5.6, 11.12 and 13.14. Similarly, the ring of six chromosomes in ^{h}*Hookeri.curvans* contains, as ^{h}*Hookeri* chromosomes, 7.8 and 9.10 with one of 5.6, 11.12 and 13.14. But since *velans.curvans* has a ring of fourteen chromosomes replacing the rings of eight and six in ^{h}*Hookeri.curvans*, it follows that ^{h}*Hookeri* 5.6 and 7.8 are in separate rings in ^{h}*Hookeri.curvans*. Inspection of the known possibilities for *flectens* chromosomes shows that the second chromosome in common between *flectens* and *curvans* must come from (1) 8.9 like *flectens* (*b*); (2) the 7.10 or 8.9 exchange with 11.12 or 13.14 and be one of 9.14, 10.12, 10.13 and 7.12 like *flectens* (*a*) or (*c*); or (3) 5.11 like *flectens* (*c*). The three *curvans* chromosomes in the ring of six with ^{h}*Hookeri* are therefore one of the following combinations:

8.9	7.11	10.12	(A)	7.10	8.11	9.12	(E)
8.9	7.12	10.11	(B)	7.10	8.12	9.11	(F)
8.9	7.13	10.14	(C)	7.10	8.13	9.14	(G)
8.9	7.14	10.13	(D)	7.10	8.14	9.13	(H)

But *curvans* cannot have any chromosomes like *rigens*, therefore (B) with 7.12 and (H) with 8.14 are impossible. Secondly, (E) and (F) would not permit a second chromosome like *flectens*, and are therefore impossible. Thirdly, (C) is impossible, as it would give a ring of four chromosomes with 8.14 and 9.10 of *rigens*. Lastly, (D) is impossible, as it would give a pair (10.13) with *gaudens* (*a*) and (*b*) or a ring of four chromosomes (8.14–*14.7*–7.9–*9.8*) with *gaudens* (*c*) and (*d*). The possibilities in which 5.11 would be the second pair with *flectens* (*c*) are all included in the arrangements which include (G). The total number of possibilities for *curvans* are therefore thirty-two. We may proceed to write all of them down and test their fit with the three *flectens* formulae and the nine s-*albicans* formulae:

								Fits *flectens*	s-*Albicans* fitting *curvans* and *flectens*
8.9	7.11	10.12	1.4	2.5	3.13	6.14	(*a*)	(*a*), (*b*)	(*j*), (*n*)
					3.14	6.13		None	
				2.6	3.13	5.14		None	
					3.14	5.13	(*b*)	(*a*), (*b*)	(*b*), (*m*)
				2.13	3.5	6.14	(*c*)	(*a*), (*b*)	(*g*), (*l*)
					3.6	5.14		None	
				2.14	3.5	6.13		None	
					3.6	5.13	(*d*)	(*a*), (*b*)	(*b*), (*k*)
			2.3	1.5	4.13	6.14	(*e*)	(*a*), (*b*)	(*a*), (*b*)
					4.14	6.13		None	
				1.6	4.13	5.14		None	
					4.14	5.13	(*f*)	(*a*), (*b*)	(*a*)
				1.13	4.5	6.14	(*g*)	(*a*), (*b*)	(*a*), (*b*)
					4.6	5.14		None	
				1.14	4.5	6.13		None	
					4.6	5.13	(*h*)	(*a*), (*b*)	(*a*)
7.10	9.14	8.13	1.4	2.5	3.11	6.12		(*c*)	None
					3.12	6.11		(*a*)	None
				2.6	3.11	5.12		(*a*)	None
					3.12	5.11		None	
				2.11	3.5	6.12		(*c*)	None
					3.6	5.12	(*j*)	(*a*)	(*a*)
				2.12	3.5	6.11		(*a*)	None
					3.6	5.11		None	
			2.3	1.5	4.11	6.12		(*c*)	None
					4.12	6.11		(*a*)	None
				1.6	4.11	5.12		(*a*)	None
					4.12	5.11		None	
				1.11	4.5	6.12		(*c*)	None
					4.6	5.12		(*a*)	None
				1.12	4.5	6.11		(*a*)	None
					4.6	5.11		None	

It follows that, since the s-*albicans* formula (s-*albicans* (*o*)) that fits *flectens* (*c*) will fit none of the *curvans* formulae that fit *flectens* (*c*), s-*albicans* (*o*) and *flectens* (*c*) are impossible. *Flectens* is therefore one of

$$1.4\quad 2.3\quad 5.7\quad 11.13\begin{array}{l}\nearrow 6.8\quad 9.14\quad 10.12\quad (a)\\ \searrow 6.10\quad 8.9\quad 12.14\quad (b)\end{array}$$

We know also that these two *flectens* formulae are fitted only by *gaudens* (*a*) and (*b*); *gaudens* (*c*) and (*d*) are therefore impossible and *gaudens* is one of

$$1.2\quad 5.6\quad 7.12\quad 10.13\quad 8.14\begin{array}{l}\nearrow 3.11\quad 4.9\quad (a)\\ \searrow 4.11\quad 3.9\quad (b)\end{array}$$

Lastly, only nine of the tentative *curvans* formulae will fit s-*albicans* and *flectens* formulae that are mutually fitted. A comparison of the mutual fitting of *curvans*, s-*albicans* and *gaudens* possibilities serves to eliminate two more of the s-*albicans* possibilities: (1) s-*albicans* (*n*) fits *curvans* (*a*); the latter is fitted by *gaudens* (*b*), which does not fit s-*albicans* (*n*). (2) s-*albicans* (*l*) fits *curvans* (*c*); the latter is fitted by *gaudens* (*a*), which does not fit s-*albicans* (*l*).

Further eliminations of the various possibilities for *flectens*, s-*albicans*, *curvans* and *gaudens* may be made on the basis of the breeding results described by Renner (1933). *Flectens*.*flavens* has a ring of six chromosomes, a ring of four and two pairs of chromosomes with 1.4 and 2.3 as the pairs. *Flectens* carries **Fl** (bent stem tip) and **M** (red-margined leaf), while *flavens* carries **fl** (erect stem tip) and **m** (non-margined leaf). In *flectens*.*flavens* **Fl** and **M** segregate independently of one another. *Flectens*.*velans* has a ring of six chromosomes and two rings of four chromosomes, with 1.4 and 2.3 of *flectens* and 1.2 and 3.4 of *velans* making one of the rings of four chromosomes. *Velans* carries **fl** and **m** and in *flectens*.*velans* **Fl** and **M** are linked and do not segregate from one another. Since **Fl** and **M** are linked in segregation in *flectens*.*velans* and are not linked in *flectens*.*flavens*, it follows that one of them is in 1.4 and the other in 2.3 of *flectens*.

From the cross s-*albicans*.*flectens* × *suaveolens* Renner (1933) obtained a bent-tipped **Fl fl** s-*albicans*.*flavens*, and this in F_2 segregated **fl fl** s-*albicans*.*flavens* and **Fl Fl** s-*albicans*.*flavens*. Now s-*albicans*.*flavens* has a ring of twelve chromosomes and a pair, the latter being 1.4. Therefore **Fl** is in 1.4 and **M** is in 2.3.

Flavens.*curvans* has two rings of six chromosomes and a pair; the latter is either 1.4 or 2.3. *Curvans* carries **M** (red-margined leaf), **Cu** (bent stem tip) which is probably homologous with **Fl**, and **b** (narrow leaf), while *flavens* carries **m**, **cu** (erect stem tip) and **B** (broad leaf) which is known (Renner 1933) to be on chromosome 7.8. In the F_2 from *flavens*.

curvans, **m** and **M** were found to have segregated from each other, both were obtained in homozygous condition and therefore both are alethal and neither is linked to a lethal. Also, **B** segregated from **b**; **B** is in 7.8 and is therefore in one of the rings of six in *flavens*.*curvans*. Although **BB** homozygotes may be obtained they usually have defective leaves and flowers, since **B** is linked in this combination with a factor **def** which produces leaf and flower defects. But **b b** homozygotes are lethal, either because **b** itself is lethal or because it is linked with a lethal in *flavens*.*curvans*. Therefore **M** is in a pair or else in the ring of six that does not contain **B b**. This second ring of six contains 5.6, of *flavens*, which carries **Sp** (pointed bud and leaves); this factor is lethal when homozygous. Now if 2.3 of *flavens* is not in a pair in *flavens*.*curvans* it must be in the ring of six chromosomes that includes 5.6. Consequently, if 2.3 is in the ring of six **m** would be linked in segregation with the lethal **Sp** and therefore unobtainable in homozygous condition. But we know already that **m m** homozygotes are viable. Therefore **m** cannot be lethal or linked to a lethal (in particular, **Sp**) and must be in a free pair of chromosomes, namely 2.3. Therefore *curvans* has 2.3 but not 1.4.

These considerations show that the five *curvans* formulae with 1.4 are impossible and therefore that *curvans* is one of

2.3	8.9	7.11	10.12	1.5	4.13	6.14	(*e*)
				1.6	4.14	5.13	(*f*)
				1.13	4.5	6.14	(*g*)
				1.14	4.6	5.13	(*h*)

Only two s-*albicans* formulae will fit these four *curvans* formulae, consequently s-*albicans* is one of

1.4	8.9	5.7	11.13	10.12	2.14	3.6	(*a*)
				2.10	3.12	6.14	(*b*)

b-Albicans (the egg complex of *biennis*).

All the data upon which the above two formulae for s-*albicans* have been inferred may be duplicated for b-*albicans*, as follows:

b-*albicans*.^{h}*Hookeri*	14	Hoeppener and Renner 1929; Cleland and Blakeslee 1930.
b-*albicans*.*flavens*	12, 2	Cleland 1928.
b-*albicans*.^{h}*purpurata*	12, 2	C. unpub.
b-*albicans*.^{h}*blandina*	14	C. unpub.
b-*albicans*.*flectens*	6, 2, 2, 2, 2	Renner 1933, 1938.
b-*albicans*.*curvans*	6, 4, 2, 2	Cleland 1928.
b-*albicans*.*gaudens*	8, 6	Cleland 1928; Håkansson 1930.

In every case the configuration observed is the same as in the corresponding complex combination involving s-*albicans* in place of b-*albicans*. It follows that b-*albicans* is one of the same two formulae that are possible for s-*albicans*.

Renner (1938) has obtained from *O. biennis* × *flavens.curvans* a combination, b-*albicans*.**b** *flavens*, which contains a complex, **b** *flavens*, that is a mixture of four *flavens* chromosomes, 1.4 + 2.3 + 5.6 + 13.14, and the triplet, 8.9 + 7.11 + 10.12, from *curvans* that brings in the narrow leaf factor, **b**, in place of the *flavens* broad-leaf factor, **B**, that is in chromosome 7.8 of the triplet 7.8 + 9.10 + 11.12. Renner states that the combination b-*albicans*.**b** *flavens* has a ring of eight chromosomes and three pairs. Comparison of the formula for **b** *flavens* with the two possibilities for b-*albicans* shows that only one of the latter could give the necessary three pairs with **b** *flavens*:

b-*albicans* is 1.4 8.9 5.7 11.13 10.12 2.14 3.6 (*a*)

This b-*albicans* formula fits only one of the two remaining *gaudens* formulae:

Gaudens is 1.2 5.6 7.12 8.14 10.13 3.11 4.9 (*a*)

Therefore *blandina*-A is

1.2 5.6 7.10 8.13 9.14 3.11 4.12 (*a*)

Also *gaudens* (*a*) will fit only one of the two possible s-*albicans* formulae, namely, the one that has the same structure as b-*albicans*. We have therefore proved, what has long been suspected, that *albicans* from *biennis* is similar in the arrangement of its end-pairing chromosome segments to the *albicans* from *O. suaveolens*. There is now no longer any need to differentiate between the source of the *albicans* complex when analysis of the present type is being conducted.

We know also that *curvans.gaudens* has a ring of fourteen chromosomes (Renner 1938). Only two of the four remaining *curvans* formulae will give such a configuration with the *gaudens* structure that has been inferred. Therefore *curvans* is one of

$$2.3 \quad 8.9 \quad 7.11 \quad 10.12 \left\langle \begin{array}{llll} 1.5 & 4.13 & 6.14 & (e) \\ 1.14 & 4.6 & 5.13 & (h) \end{array} \right.$$

Renner (1938) has described the behaviour of a mixed complex, **B** *curvans*, consisting of four *curvans* chromosomes and three *flavens* chromosomes. It has the *flavens* chromosomes 7.8, 9.10 and 11.12 instead of the three *curvans* chromosomes 8.9, 7.11 and 10.12. From the cross *O. biennis* × *curvans.flavens*, Renner obtained the combination *albicans*.**B** *curvans* and found that it had a ring of eight chromosomes and a ring of six. The three chromosomes that **B** *curvans* received from *flavens* make a chain of seven chromosomes with four of the *albicans* chromosomes, namely (*flavens*, i.e. **B** *curvans*, chromosomes in italics), 5.7–*7.8*–8.9–*9.10*–10.12–*12.11*–11.13. Therefore **B** *curvans* must have 5.13, from *curvans* possible formula (*h*), to complete the ring of eight chromosomes given with *albicans*. If the other possible set of four *curvans* chromosomes, those in formula (*e*), were in the **B** *curvans* mixed complex, *albicans*.**B** *curvans* would have a ring of ten chromosomes and a ring of four. This does not fit the observations. It follows that *curvans* has the second (*h*) of the two possible formulae:

Curvans is 1.14 2.3 4.6 5.13 7.11 8.9 10.12

Flavens.*flectens* has a ring of six chromosomes, a ring of four and two pairs. The pairs are 1.4 and 2.3 and carry respectively the factors **Fl** (in 1.4) and **M** (in 2.3) from *flectens* and **fl** and **m** from *flavens* (Renner 1933). Also, *flectens* carries the factor **lor** (*loreus*) which is recessive to the homologue **Lor** carried by *flavens*. When homozygous, **lor** is a semilethal, the plants being crippled dwarfs with narrow leaves. In the F_2 from *flavens*.*flectens*, **lor lor**, **Lor lor** and **Lor Lor** plants are obtained; the segregation of **lor** from **Lor** is independent of those of **Fl fl** and of **M m** which are also independent of one another. Therefore, **Lor** and **lor** are located in the chromosomes which make up the ring of four or the ring of six. Renner (1933, p. 235) has shown that **Lor lor** plants have the same configuration as the F_1, namely, 6, 4, 2, 2. All plants that are homozygous for **lor** or **Lor** have 6, 2, 2, 2, 2 whether or not they are homozygous for **M** or **m** or **fl**. Consequently the **lor** factor lies in a ring of four chromosomes in *flavens*.*flectens*. This ring of four is constituted as follows: *5.7*–7.8–*8.6*–6.5 or *11.13*–13.14–*14.12*–12.11 (*flectens* chromosomes in italics), according to whether *flectens* formula (*a*) or (*b*) is correct. In the first case, **Lor**, the factor that lies in *flavens* chromosomes would be linked with the lethal **Sp** in chromosome 5.6 of *flavens*. In the first case therefore **Lor** would be unobtainable in the homozygous condition; this construction of the ring of four must be impossible:

Flectens is 1.4 2.3 5.7 11.13 6.10 8.9 12.14 (*b*)

Rubens (a complex of *O. biennis*) is known to have 1.2 and 5.6 (Cleland 1937):

rubens.$^{\mathrm{h}}$*Hookeri*	10, 2, 2	Cleland 1928.
rubens.$^{\mathrm{h}}$*blandina*	10, 2, 2	C. unpub.

rubens.ʰ*purpurata*	10, 2, 2	C. unpub.
rubens.*rubricalyx*-α	10, 2, 2	C. unpub.
rubens.*rigens*	6, 2, 2, 2, 2	C. unpub.; Renner 1938.
rubens.*albicans*	8, 6	Cleland 1926.
rubens.*curvans*	14	Renner 1938.

This complex has two chromosomes like *rigens*, but not like ʰ*Hookeri*, i.e. two of 7.12, 8.14 and 11.13. There is no pair with *albicans*, therefore 11.13 is impossible; *rubens* has 7.12 and 8.14. The remaining chromosomes are an interchange arrangement of the *rigens* chromosomes 3.4, 9.10 and 11.13. There are eight possibilities for this arrangement:

1.2 5.6 7.12 8.14	3.9	4.11	10.13	(i)
		4.13	10.11	(ii)
	3.10	4.11	9.13	(iii)
		4.13	9.11	(iv)
	3.11	4.9	10.13	(v)
		4.10	9.13	(vi)
	3.13	4.9	10.11	(vii)
		4.10	9.11	(viii)

Of these, (ii) and (vii) are impossible, for they would give a ring of four chromosomes with 7.10 and 11.12 of ʰ*blandina* and ʰ*purpurata*; formulae (iii) and (vi) are impossible, for they would give a ring of four chromosomes with ʰ*blandina* 8.13 and 9.14; and formulae (i) and (viii) would give a ring of fourteen chromosomes with *albicans* and are therefore impossible. Of the two remaining possibilities only formula (v) gives a ring of fourteen chromosomes with the *curvans* formula. The other formula (iv) gives a ring of ten and a ring of four chromosomes with the *curvans* formula. Therefore *rubens* is

1.2 5.6 7.12 8.14 3.11 4.9 10.13

We have therefore been able to demonstrate that *rubens* has an arrangement of the end-segments of its chromosomes exactly like that of *gaudens*.

DISCUSSION

In a paper which has sought chiefly to provide a firm cytological basis for genetical and evolutionary studies that are in progress no important general conclusions may be expected. The evolution of the genus, at least in its more recent phases, has involved the establishment of a balanced lethal system associated with and maintained by a system of structural hybridity that is mainly interchange in character. An investigation of the chromosome constitution of a large number of complexes should indicate in a general way the approximate order of the different interchanges which have occurred in the differentiation of the races. The precise determination of the various steps in the change require an exact set of definitions of the parts that are subject to the change. These are provided by the analyses outlined above. Prof. R. E. Cleland has indicated (*in litt.*) that he has made a consistent analysis of a large set of data including relatively few of the complexes employed in the present analysis. Moreover, the two series are mutually consistent, showing that there can be no particular error anywhere in the enormous mass of data that has been accumulated and sifted. It should now be possible to make a more rapid progress in further analyses.

The identity of structure in the pair of complexes *gaudens* (of *Lamarckiana*) and *rubens* (of *biennis*) and the pair of *albicans* complexes in *biennis* and *suaveolens* shows that the three European species in which they occur are related in origin, though *Lamarckiana* and *suaveolens* actually have no complex in common. The probabilities are that *Lamarckiana* and *suaveolens* are derived from a cross or crosses between *biennis* and one or two unknown forms. There can be little doubt that *biennis* is one parent because races similar to it are known wild in North America, whereas *Lamarckiana* and *suaveolens* are not known there except in gardens or in situations where they have obviously escaped from cultivation. The species *Lamarckiana* and *suaveolens* must have originated after the introduction of *biennis* to Europe, that is, in the post-Columbian period, namely, within the last 500 years. Even so we know that *Lamarckiana* and *suaveolens* have been in existence for at least 143 and 136 years respectively. During those periods there has been no major differentiation in the structure of the chromosomes of the *gaudens* and *rubens* complexes or of the *albicans* complexes of *biennis* and *suaveolens*. The complexes are relatively stable in structure; new interchanges that are evolutionarily successful do not arise with great frequency.

This conclusion is in agreement with the growing evidence that not all the possible interchanges have occurred in nature. So far as analyses have gone, *Oenothera* complexes are structurally much more like one another than they should be if the present population is a random selection of all the possible interchanges that could occur. It has already become clear from the investigations of Cleland (1935) that structural arrangements like those of the Californian races referred to *Hookeri* and *franciscana* are relatively primitive. Calculation from published and unpublished data shows that complexes which are structurally more like California-type complexes are also more like all other complexes in their structure than is an average complex. Considerations of this kind are likely to help in the disentangling of a complex phylogeny, the trends of which could only be hinted at with our present fragmentary information.

The identification of the chromosome structure will also aid the genetic mapping of the chromosomes. We have already seen that breeding results and cytological observations may supplement one another in determining the structure of the chromosomes. At the same time the combination of such data serves to locate particular genes in particular chromosomes or even in a particular arm of a chromosome (Emerson and Sturtevant 1932; Renner 1933). It is therefore now possible to enter on a map the approximate location of several genes. The general form of such a map (figure 1) is a star, as was first pointed out by Darlington (1931), with the arms representing the end-pairing segments of the chromosomes and the dotted circle the differential segments of the chromosomes. Zero locus on the chromosome map is at the proximal end of each arm. It is a remarkable feature of *Oenothera* that most known genes are tightly linked to the differential segments. The majority show only 1 or 2 % recombination with these segments. There are only two exceptions to this phenomenon, namely, the major genes controlling flower size (Co giving small flowers) and the gene *brevistylis* (**br**) that shortens the style, deforms the stigma and also produces characteristic effects in other organs of the plant. Both these genes segregate independently of the differential parts of the complexes (Renner 1931; Langendorf 1930), and therefore they are situated at least fifty map units out along one or other of the pairing segments of the chromosomes (Emerson and Sturtevant 1932). Indeed, every arm of the map must be fifty units long at the least, since each pairing segment forms an average of more than one chiasma at the end of pachytene (Catcheside 1932).

The data summarized in the map of *O. Lamarckiana* (figure 1) are compiled from data in the papers of de Vries, Renner, Oehlkers, Cleland, Emerson and Sturtevant, Shull and myself. Some parts of the map are speculative and will no doubt not stand further tests. However, the map does express in graphical form our present knowledge of the genetic structure of this plant.

The map is of course only a rough approximation which takes no account of the distribution of interstitial segments (between the locus of interchange and the centromere) and of differential segments (between two loci of interchange in the same chromosome). We do not know how complex the structural hybridity is in these plants, but the presence of differential segments may be detected by the effects they produce. Occasional pairing and crossing-over of homologous differential segments, which are several chromosomes apart in the ring, result in a new complex constructed of parts of each of the parent complexes. One of the chromosomes in the new complex is unlike any chromosome of the old complexes, since it consists

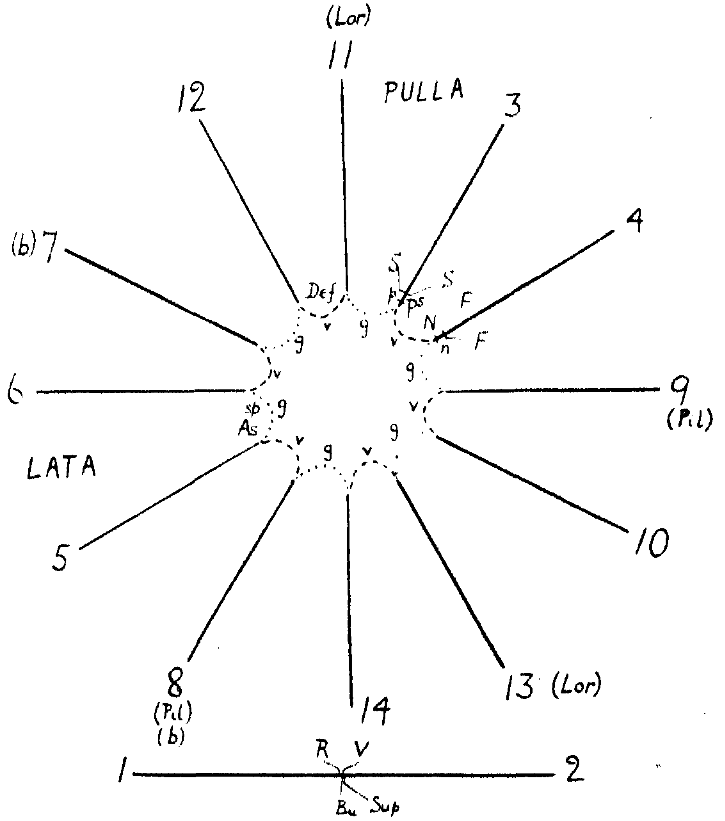

FIGURE 1. The map of chromosome 1.2 is a straight line; it has four known gene loci, all close together near the middle of the chromosome. The map of the remaining twelve chromosomes is a composite star, each of whose twelve arms represents a pair of end-segments. Differential segments are represented for all chromosomes in the absence of contrary information; they belong alternately to *velans* (broken line, *v*) and *gaudens* (dotted line, *g*). Chromosomes 5.6 and 3.11 are known to be the extra chromosome in two trisomics, *lata* and *pulla* respectively. Definitely located genes are known for segments 3 and 4; others are not certainly allocated and are given in brackets.

of parts of two old chromosomes, namely, the two whose differential segments paired. Three such new complexes derived from *O. Lamarckiana* have been analysed in the present study, namely, ^{h}*blandina*, *latifrons* and *rubricalyx-α*. The new chromosomes that they contain should indicate the possible positions in the parent complexes of the differential segments that produced them. Unfortunately the problem is immediately complicated by the fact that each of the new complexes contains more than one new chromosome. Thus ^{h}*blandina* has 7.10, 8.13 and 9.14, *latifrons* has 7.10 and 9.13, and *rubricalyx-α* has 7.14 and 8.13, none of which chromosomes are present in either *velans* or *gaudens*. This means that pairing and crossing-over has taken place in at least two different pairs of differential segments either at the same time or separately. Possibly the second structural change may have occurred in each case after the new complex had appeared from *Lamarckiana*. If that is so, it is indeed curious that all three of these complexes should have changed their structure. Further, I have studied four strains of *rubrinervis*-like interchange mutants, all of which have a new complex with the same structure as *rubricalyx-α* and all of which are of independent origin from *Lamarckiana* or a trisomic thereof. The subject requires further detailed analysis that cannot be pursued at this point.

SUMMARY

The fourteen end-pairing segments of *Oenothera* chromosomes have been defined (cf. pp. 514 and 517 for summaries of definitions) in terms of their property of pairing at meiosis and the ways in which they are combined

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Key to gene symbols on figure 1

As synaptic *v.* asynaptic (**as**) chromosomes at meiosis.
b narrow *v.* broad (**B**) leaves.
Bu flat *v.* bullate (**bu**) leaves.
Def normal *v.* defective (**def**) flowers.
F flat *v.* revolute (**f**) leaves.
Lor normal *v.* loreus (**lor**), i.e. extremely narrow leaves.
N tall *v.* nanella (**n**) stature.
P^{s} red striped buds } Two members of a series of multiple allelomorphs.
p green buds }
Pil glabrous *v.* hairy (**pil**) flower buds.
R red *v.* white (**r**) midribs to leaves.
S yellow *v.* sulphur (**s**) flower colour.
sp blunt *v.* pointed (**Sp**) leaves and buds.
Sup single *v.* double (**sup**) flowers.
V yellow *v.* old gold (**v**) flower colour.

in the chromosomes of different gametic complexes. The comparison of a number of different complexes has led to the following conclusions concerning their structure in respect of the end segments of the chromosomes:

$^{\mathrm{h}}$*Hookeri*	1.2	3.4	5.6	7.8	9.10	11.12	13.14
flavens	1.4	2.3	5.6	7.8	9.10	11.12	13.14
velans	1.2	3.4	5.8	6.7	9.10	11.12	13.14
excellens $^{\mathrm{h}}$*franciscana* $^{\mathrm{h}}$*purpurata*	1.2	3.4	5.6	7.10	8.9	11.12	13.14
$^{\mathrm{h}}$*blandina*	1.2	3.4	5.6	7.10	8.13	9.14	11.12
blandina-A	1.2	3.11	4.12	5.6	7.10	8.13	9.14
blandina-B	1.4	2.3	5.6	7.10	8.13	9.14	11.12
latifrons	1.2	3.4	5.6	7.10	11.12	8.14	9.13
rubricalyx-α $^{\mathrm{h}}$*decipiens*	1.2	3.4	5.6	7.14	8.13	9.10	11.12
rigens	1.2	3.4	5.6	7.12	8.14	9.10	11.13
gaudens *rubens*	1.2	3.11	4.9	5.6	7.12	8.14	10.13
flectens	1.4	2.3	5.7	6.10	8.9	11.13	12.14
albicans	1.4	2.14	3.6	5.7	8.9	10.12	11.13
curvans	1.14	2.3	4.6	5.13	7.11	8.9	10.12

The value of the analysis in relation to evolutionary and genetical studies of these plants is pointed out. The three European species *Lamarckiana*, *suaveolens* and *biennis* are related in origin; each of the first two of these has a complex in common with *biennis*, but a different one in each case. The common complexes are identical in gross structure and very similar in gene content. A preliminary genetic map of *Lamarckiana* has been drawn and a complication in the origin of new alethal complexes from *Lamarckiana* laid bare.

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* This paper had not been seen when the present paper was written; several conclusions reached agree with the results given here.

Studies on the transmission of sugar-beet yellows virus by the aphis, *Myzus persicae* (Sulz.)

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Previous studies on the relationships between plant viruses and their insect vectors have been carried out with viruses which are easily mechanically transmissible and whose vectors lose their infectivity within a few hours of removal from the source of infection. This type of virus has been called (Watson and Roberts 1939) *non-persistent*, for it was observed that the property of non-persistence of the virus in the vector was associated with other properties in which viruses of this type resemble each other, and differ from those viruses whose vectors retain their infectivity for long periods, namely, the *persistent* viruses. It seems that these differences must lie in the nature of the viruses themselves, for viruses of both types can be transmitted by the same vector.

Sugar-beet yellows virus (Petherbridge and Stirrup 1935) seems to be a member of the *persistent* class, for its vector, ***Myzus persicae***, the same insect as was used in previous work on *non-persistent* viruses (Watson 1936, 1938; Watson and Roberts 1939), remains infective for several days after removal from the source of infection (Roland 1939). Also it is not transmissible mechanically by any of the usual methods (Quanjer 1934, 1936). The present paper, therefore, describes some studies on the vector-virus relationships of this virus by the methods which have been used previously only on the *non-persistent* types.

MATERIALS AND METHODS

The virus used in most of these experiments was propagated from infected sugar-beet leaves kindly sent by Professor Quanjer from Wageningen, but the symptoms were indistinguishable, except in intensity, from those produced in plants to which infection was transmitted by aphides, from sugar-beet and mangold leaves collected from six different counties in England. When tested, the properties of the viruses from these different sources were also found to be identical, the differences in the intensity of symptoms being reproducible over a succession of inoculations. This suggested that the viruses collected were the same as that obtained from Wageningen, but that strains of varying virulence existed. As the symptoms produced in the glasshouses are in any case slightly different from the appearance of the disease in the field, a short description of the two main types is given.

The milder strains, which were obtained from Wageningen, Woburn (Beds), Peterborough (Lincs), Leeds (Yorks) and Tunstall (Suffolk), show their first symptoms about 10–30 days after inoculation, as small yellowish or orange-yellow patches, often at the site of penetration by the infective insects. These lesions spread, causing at first asymmetrical, and later complete, discoloration of the leaf, which eventually withers. Frequently the general withering of the leaf can be traced back to necrotic patches which appear in the yellowed areas and coalesce causing complete necrosis and death. During the winter months the leaves wither very rapidly after the yellow symptoms appear, and as they show symptoms in succession from the older leaves inwards, it frequently happens that an infected plant shows no symptoms at all, except for withered leaves remaining attached at its base. In the winter months, therefore, the more virulent strains were used. These were obtained from Rothamsted (Herts) and Hornsea (Yorks). Their first symptoms, which in good weather appear 8–12 days after inoculation, are "vein clearing" of medium-aged leaves followed later by the appearance of local symptoms on the older leaves. The vein clearing may be very bright yellow, or have a necrotic appearance caused by collapse of the cells which lie immediately over the veins. It generally starts at the distal ends of the leaves, and may spread all over the leaf surface or remain more or less localized. In the summer months even the weaker strains produce a localized vein clearing in a few plants, but it is not so severe, or so common as with the others.

Clearing of the veins and "net-necrosis" occur as symptoms of sugar-beet yellows in the field as well as in the glasshouse, especially at Rothamsted. Even in this form it is quite easy to distinguish from the disease caused by sugar-beet mosaic virus which shows blotchy green vein-banding of all the younger leaves, whereas sugar-beet yellows symptoms never affect the very youngest leaves.

"Gummosis" of the phloem, described by Quanjer (1934) as a characteristic symptom of sugar-beet yellows, has not been found in the glasshouse plants, even in very well-established infections.

The methods of culturing and handling the insects used in the following experiments were similar to those already described for *Myzus persicae* (1936, 1938), but as the probability of infection by a single aphis per plant was found to be exceedingly low, the experiments were all carried out using five aphides for each healthy plant tested. The sugar-beet plants used for receiving the aphis transmission were at the stage when they had two leaves about 1–1½ in. long. The age of these plants varied considerably with the season, but was generally from 3 to 5 weeks. The variety used was *Kleinwanzleben E.*

The experiments were designed to test the infectivity of the aphides after varying times of feeding on infected and healthy plants. They may conveniently be divided into two groups. The first are those experiments in which each aphis group was tested for its performance on one healthy plant only, and in the second each group was tested on a succession of two or more consecutive healthy plants.

A. INFECTIVITY OF APHIDES TESTED ON SINGLE HEALTHY PLANTS

(1) *Effect of feeding time on the infected plants*

In this experiment with the Wageningen strain of the virus, aphides were starved for a few hours, then placed on infected leaves for 5 min.

(allowing 2–3 min. actual feeding), 1 hr. or 18 hr. Aphides which were observed to be feeding were then transferred to healthy seedlings and allowed to feed for 24 hr. The experiment was repeated three times using fifteen plants for each treatment on each occasion. No infections were obtained from the 2 min. feedings. The 1 hr. feedings gave 11 % and the 18 hr. feeding 69 % infection. There was thus considerable increase in infectivity with increasing feeding times on the infected plants, and the chance of obtaining infective insects from very short infection feeding periods seemed to be very small.

(2) *Effect of feeding time on the healthy plants*

There were two series of tests carried out on the effect of feeding time on the healthy plant. Constant infection feeding of about 18 hr. was given in both series. In the first, using the Wageningen strain of virus, the feeding times on the healthy plants were 20, 30, 40, 90 and 180 min. In the second the Rothamsted strain was used and the times were 5, 10, 20 and 40 min.

The results are given in table 1.

TABLE 1. PERCENTAGE INFECTIVITY FOR VARYING FEEDING TIME ON HEALTHY PLANTS AFTER CONSTANT INFECTION FEEDING. W. = WANGENINGEN STRAIN, FIVE PLANTS PER TREATMENT REPEATED ON SEVEN OCCASIONS. R. = ROTHAMSTED STRAIN, FIVE PLANTS PER TREATMENT REPEATED ON FIVE OCCASIONS

		Feeding time on healthy plants (min.)						
		5	10	20	30	40	90	180
Percentage infection	W.	—	—	17	23	31	58	61
	R.	8	24	32	—	64	—	—

The infectivity of the aphides increased rapidly with increasing infection-feeding time up to about 90 min., and then more slowly. The more virulent strain used in the second series of tests gave a considerably higher level of infectivity, but the rate of increase seemed to be about the same as in the first, i.e. infectivity doubled between 20 and 40 min. It will be seen from later experiments that though increase in infectivity becomes slower for the longer feeding time it does in fact continue for several hours.

(3) *Interaction between feeding times on infected and healthy plants*

This experiment was arranged on a factorial design using five times of feeding on the infected plants and three times of feeding on the healthy plants. The results and treatments are shown in table 2.

As in the previous experiments, increasing the time spent on both infected and healthy plants increased the probability of infection, but if the data are expressed graphically, the shape of the curves suggests that infection might have been obtained for even shorter total feeding times, or else that trials over a shorter range of feeding times might demonstrate the existence of a threshold period below which the insects were incapable of completing the transmission of the virus. Such a threshold period has been described for many viruses whose other properties resemble those of sugar-beet yellows virus. This is referred to as the "latent" or "incubation" period, for with most persistent viruses it seems to be so important and prolonged that it was thought to be the time taken for a fundamental and necessary change in either vector or virus.

A second factorial experiment was carried out using shorter feeding periods on both plants. The treatments and results are given in table 3.

TABLE 2. NUMBER OF PLANTS INFECTED FOR VARYING FEEDING TIMES ON HEALTHY AND INFECTED PLANTS. FIVE PLANTS PER TREATMENT. REPEATED ON TEN OCCASIONS. WAGENINGEN STRAIN

Feeding time on healthy plants (hr.)	Feeding time on infected plants (hr.)						Mean percentage infection
	½	2	6	18	24	Total	
1	3	18	23	22	25	91	36
4	7	24	33	34	34	132	53
20	8	33	36	41	41	159	64
Total	18	75	92	97	100	382	
Mean percentage infection	12	50	61	65	66		

TABLE 3. EFFECT OF SHORT FEEDING TIMES ON INFECTED AND HEALTHY PLANTS. FIVE PLANTS PER TREATMENT; REPEATED ON FIVE OCCASIONS. ROTHAMSTED STRAIN

Feeding time on healthy plants (min.)	Feeding time on infected plants (min.)			
	7	15	30	Total
7	0	0	2	2
15	0	1	1	2
30	1	2	3	6
Total	1	3	6	10

The shortest total feeding time in which an infection was obtained was 30 min., but 7 min. on either infected or healthy plants caused infection when the other feeding was more prolonged. The results are precisely what might be expected from those given in table 2 except that the general level of infectivity, considering the shortness of the feeding times, is higher. This, as in table 1, is due to the use of the more virulent Rothamsted strain of the virus.

There is no indication of a lower limit of time for the feeding periods, either separately or in combination, below which infectivity could not be obtained provided sufficient trials were made. As the presence of such a threshold or incubation period is so common that it is considered by most workers to be diagnostic of the type of virus for which it is found, increasing difficulty is experienced in deciding which type of insect-transmitted virus is represented by sugar-beet yellows virus. It must be concluded either that this virus is a type different from any which has yet been described, for it certainly cannot be included with the *non-persistent* viruses, or else that the possession of an incubation period is not necessarily diagnostic of the *persistent* type.

Whether sugar-beet yellows virus is in fact unique in its behaviour in relation to its insect vector remains to be proved by comparison with other *persistent* types, especially those with the same, or similar vectors, such as leaf-roll virus of potato. However, some results obtained with curly-top virus of sugar-beet, particularly by Severin (1931) and Freitag (1936), present some interesting grounds for comparison.

Severin showed (1931) that on rare occasions infections could be obtained with curly-top virus after the vector *Eutettix tenellus* had fed for only 10 min. on infected and healthy plants respectively. As curly-top has been variously reported as having an "incubation period" of "from 6 to 24 hr." and "from 1 to 44 days", Severin regarded the early infections as anomalous and suggested two possible explanations for them. One was that a few insects were capable of causing mechanical infections produced by contamination of the stylets, as transmission of the *non-persistent* viruses is commonly supposed to be effected. In support of this hypothesis he showed, by feeding non-viruliferous insects on the washings from the stylets of viruliferous insects, that these stylets were, in fact, contaminated with the virus. The other suggestion, put forward by Swezy (1930), was that a pathological condition of the oesophageal valve occasionally enabled the virus to be regurgitated directly into the plant, without its having to undergo the period of circulation through the body of the insect which they considered to take place during the normal "incubation period".

Severin adduced these arguments because he believed that the time in which the short feeding infections took place was insufficient for such a circulation of the virus to have occurred. However, Severin's results could equally well be interpreted as showing that the infectivity of the vectors increased steadily with increasing feeding time on infected and healthy plants, in a manner similar to that exhibited by the vectors of yellows virus. In his comparative experiments the feeding times were not continued beyond a total of 4 hr., but if the early infections were caused mechanically this time should have been sufficient to give at least some indication of containing the first of two optima of infectivity, one occurring as soon after access to the source of infection as is necessary for the contamination of the stylets and delivery of the virus to the healthy plant, and one after "normal" incubation period had elasped. There is no indication of such a double optimum, and every indication that the infectivity would continue to increase in a smooth curve. Other results obtained with curly-top virus, notably those of Freitag (1936), further indicate that this increase in infectivity may continue slowly for 5 or 10 days. This suggests that the curve for increasing infectivity of the vectors of curly-top virus may be similar in shape to that of yellows virus in that, passing through zero, it rises steeply for the first part of its course and flattens out towards its maximum, but the sequence of changes is passed through more slowly than it is for yellows virus.

Thus the data published on curly-top virus may indicate that, like yellows virus, there is strictly no period after feeding on the infected plants during which the vectors are unable to transmit the virus, but merely a period of increasing infectivity towards a maximum at which all insects capable of transmitting infection will do so. The misconception seems to have arisen because when the feeding period on the infected and healthy plants is short the probability of infection is so low that it has appeared to be negligible. It seems unjustifiable to invoke complex *ad hoc* hypotheses such as those of Severin in which infections obtained after short feeding periods are assumed to be anomalous, when the results are capable of a simpler explanation which covers both short and long feeding times.

The simpler explanation suggested is that the behaviour of the virus in response to varying feeding times on both infected and healthy plants can be explained on a purely quantitative basis. This is not a new idea, for Freitag (1936), though accepting in principle the convention of a fixed incubation period, does not believe that this entails any fundamental change in either virus or vector, and Storey has on several occasions (1939) expressed the view that the behaviour of the vectors can be explained on quantitative or mechanical grounds.

To explain the present results with yellows virus, as given in tables 1 and 2, one may start by postulating that infectivity increases with increasing feeding time on the infected plants, because the amount of virus taken up by the aphides increases with the time during which virus is available for ingestion. But the infectivity of the vectors also increases with feeding time on the healthy plants, and the relative rate of increase seems to be independent of the supposed amount of virus ingested by the insect, or the time which it has taken to ingest it. The most simple case to consider is that of an insect which has fed on the infected plant only sufficiently long to give it a single infective dose of virus. This insect may deliver its infective dose immediately on penetrating the healthy plant, or within a few minutes of so doing, or the infective dose may not reach the healthy plant for some hours. Thus over a number of infection trials there is an increase in infectivity with increasing time spent on the healthy plants, which is made up of infections caused by aphides at increasingly longer intervals from the cessation of infection feeding. In other words, the effective transmission of the virus may be delayed, and, granting this hypothesis, the results show that some delay occurs in most infection trials. The cause of this delay is unknown, but at least one suggestion put forward to account for the "incubation period" may be apposite. It may be that time is required for the virus to circulate through the body of the vectors; but if so this time must vary between limits of very short and very long periods.

At first sight the observation that the increased infectivity with increasing feeding time on the healthy plant is independent of the infection feeding time is less explicable when the results for the longer feeding times are considered. These insects presumably contain several infective doses of virus, and it would be expected that the optimum infectivity should be reached more rapidly if the delay in the development of infectivity were a true threshold instead of a time lag varying from a few minutes to many hours. However, as the infective doses of virus are taken in over a period of time, which is the assumption originally made from the behaviour of the vectors in response to feeding time on the infected plants, there is no difficulty in supposing that they are given out in the same order, and if each infective dose undergoes the period of delayed transmission characteristic of the individual insect, the results of many trials among a mixed population of insects would be similar to those actually obtained.

Alternatively, the length of the delay in development of infectivity may not be an attribute of an individual aphid; any dose of virus in any insect may be ejaculated by chance early or late in the course of feeding on the healthy plant, but the doses picked up late in the infection feeding would, on the whole, still appear later than the earlier ones. One has only to consider the case in which an infective dose picked up early in a long infection feeding might be returned later to the infected plant and thus have no opportunity of reaching the healthy plant at all. This is probably one factor which determines the slow rate of increase in infectivity with infection feeding time of 6 hr. onwards.

This explanation fits the facts so far observed for the transmission of sugar-beet yellows virus, and might apply also to curly-top of sugar-beet. They are given thus early in this paper because it is simpler to consider the behaviour of the vectors on one healthy plant only than on a succession of healthy plants, though in fact their behaviour on a succession of healthy plants seems to bear a simple relation to their behaviour on single plants. This behaviour has been explained as being due to the interaction of three factors: (1) increase in the amount of virus ingested by the aphides with increasing feeding time on the infected plants; (2) varying periods of delay in the development of infectivity at different infection trials; (3) loss of virus during and after access to the source of infection. It will be technically difficult and may be impossible to obtain direct experimental proof of the truth of these premises, though some points can be tested and are already receiving attention, but they form a convenient working hypothesis which is justified by the facts so far obtained.

B. INFECTIONS ON SUCCESSIVE HEALTHY PLANTS

The property of the *persistent* viruses, which has most strongly supported the view that the "incubation period" is an interval of multiplication of the virus in the body of the vector, is the capacity of the vectors for infecting a large number of plants in succession over a long period of time. We have shown (Watson and Roberts 1940) that this property is not entirely confined to the *persistent* type of virus, and that *non-persistent* viruses, which certainly do not multiply in the bodies of their vectors, can give a considerable number of consecutive infections for as long as they retain the capacity to infect. The capacity for causing consecutive infections, therefore, is not only poor evidence of multiplication of the virus, but is not even a good method of distinguishing between the two types. A more important consideration seems to be the length of time for which successive infections can be obtained, and the following experiments were designed to show the effect of varying feeding treatments on the efficiency and duration of this capacity, rather than to find the maximum number of consecutive infections which could be obtained. It is obvious that with groups of five aphides one cannot state with certainty that all successive infections are caused by the same individual, in fact there is strong evidence to suggest that some of them were not, but as the aphides in each group were a random selection, the efficiency of the group is presumably most frequently that of its most successful individual, assuming that the infections are local and independent, and not cumulative.

(1) *Infections obtained at two successive feedings after varying infection feeding time, and with varying feeding time on the first healthy plant*

Table 4 gives the results obtained when aphides were fed for a constant period of 20 hr. on the second of two consecutive healthy plants, the times on the first plants and the infection feeding time having been varied. The results obtained on the first healthy plants have already been given in table 2, for they were not affected by the subsequent behaviour of the aphides. The present examination of the data is to determine the effect of the previous treatments on the infectivity of the vectors at their later feeding.

As on the first plants, the infectivity of the vectors on the second plants increased with increasing infection feeding time, and the increase was most rapid for the first 6 hr. Increasing time on the first healthy plant caused increased infectivity on those plants, but decreased infectivity on the second healthy plant. In fact, the longer the aphides remained on the first plants the fewer infections they were able to cause on the second, even though the time spent on the second plants was long enough for their optimum infectivity to have been exhibited.

The behaviour of the aphides in the previous experiments where they were tested on only one healthy plant for each group was explained as being due to the interaction of three factors: (1) increase in the amount of virus ingested by the aphides with increasing infection feeding time; (2) varying periods of delay in the development of infectivity in individual aphides; (3) loss of virus during and after access to the source of infection. No modification of this hypothesis is required to account for the results given in table 4.

Increasing feeding time on the infected plants caused increase in the amount of virus available for dissemination, and extension of the range of time over which it could be disseminated. The increase in the total infectivity of the aphides at both feedings is clearly seen, and it is also apparent that more insects remain infective for long periods when the infection period was prolonged. This effect is perhaps more obvious in the next experiment (table 5) where the successive feedings were carried on to a third day, and only the aphides which had received long infection feeding retained their infectivity, although the shorter infection feeding period (4 hr.) was approaching the optimum.

TABLE 4. CONSECUTIVE INFECTIONS WITH VARYING FEEDING TIMES ON BOTH INFECTED AND HEALTHY PLANTS. FEEDING TIME ON SECOND PLANTS = 20 HR. TOTAL OF FIFTY PLANTS FOR EACH COMBINATION OF TREATMENTS

Feeding time on infected plants hr.	Infections obtained on	Feeding time on first healthy plant (hr.)			
		1	4	20	Total
½	First plant	3	7	8	18
	Second plant	1	1	1	3
	Total	4	8	9	21
2	First plant	18	24	33	75
	Second plant	21	15	2	38
	Total	39	39	35	113
6	First plant	23	33	36	92
	Second plant	30	20	9	59
	Total	53	53	45	151
18	First plant	22	34	41	97
	Second plant	36	28	9	73
	Total	58	62	50	170
24	First plant	25	34	41	100
	Second plant	32	21	8	61
	Total	57	55	49	161
Total	First plant	91	132	159	382
	Second plant	120	85	29	234
	Total	211	217	188	616

The action of the second factor, delay in the development of infectivity by the vectors, is most conspicuous when the feeding time on the first plant was only 1 hr., for then more infections were obtained at the second feeding than at the first, so that, obviously, some of the aphides could not have developed infectivity until after ceasing to feed on the first plants. Examination of the results for individual aphis groups (§ B (4)) showed that some second infections were caused by aphides which did not infect the first plant even after feeding on it for 4 hr., while a rather larger, but still small, number of insects apparently lost their infectivity during the first feeding, so that on balance there were less infections at the second than at the first feeding. This loss is the earliest manifestation of the effect of the third factor, i.e. loss of virus from the infective aphides, whose later effect is obvious in the low efficiency of the aphides on the second day of transfer to healthy plants. The same kind of result was obtained in all the experiments on successive infections.

Loss of infectivity by the aphides is shown both by the lack of increased efficiency when the infection feeding time is increased beyond 6 hr., and in the reduction in the numbers of healthy plants infected at the later successive feedings. There is nothing in the facts so far obtained to suggest that this is not completely accounted for by virus which is returned to the infected plant during the longer infection feedings, and thus "wasted", and also by that introduced into the healthy plants, which may receive many infective doses of virus though only one is accounted for in the results. Many viruses, however, exhibit the property of gradually losing their infectivity *in vitro* in expressed sap, or even in the purified state, and it is possible that some of the loss which occurs in the insects is due to this type of inactivation.

The analysis of variance of the results, for the numbers of second plants infected, given in table 4 suggests that there is an interaction between time of feeding on infected and first healthy plants. This is probably accounted for by the smallness of the increase for increasing infection feeding time when the time spent on the first plants was 20 hr., and the fact that, when the infection feeding time was only ½ hr. the number of second plants infected for the different feeding times on the first plants was uniformly low. The first is probably merely an indication that when the amount of virus in most of the aphides is nearing exhaustion, increased opportunity for its dissemination is not greatly effective in increasing their efficiency, and the second is really a problem concerned with the behaviour of individual aphides which is discussed in a later section (B (4)).

(2) *Consecutive infections after varying infection feeding times*

In this experiment the feeding time on the healthy plants was constant, and that on the infected plants varied. The arrangement and results can be seen in table 5.

As in the previous experiments less infection was obtained on the second day than on the first day for equal lengths of feeding time, and very much less on the third day. This does not agree with the results of Roland (1939), who concluded that the aphides were equally infective on the third day as on the first. His results may, however, have depended upon the number of aphides used per plant, for if the number were very large, the number of plants infected might depend more on the susceptibility of the plants than the infectivity of individual aphides.

TABLE 5. INFECTIONS OBTAINED FOR 24 HR. FEEDING ON SETS OF THREE CONSECUTIVE HEALTHY PLANTS AFTER VARYING INFECTION FEEDING TIMES. FIVE PLANTS PER TREATMENT, REPEATED ON TEN OCCASIONS. TOTAL, FIFTY PLANTS FOR EACH COMBINATION OF TREATMENTS

Infection feeding time hr.	Infections obtained on		
	First plant	Second plant	Third plant
4	31	8	0
24	39	20	2
48	35	15	1
Mean percentage infection	70	28	2

Aphides with a relatively low initial charge of virus, e.g. after only 4 hr. infection feeding, appeared to be non-infective by the third day, and their infectivity on the second day was lower than that for the 24 and 48 hr. infection feeding though there was actually little difference in the total infectivity, i.e. the amount of virus, lost by the three sets of aphides.

The increase in infectivity for increasing feeding times on the infected plants did not continue after 24 hr. In fact, there was slightly less infectivity after 48 than after 24 hr. This difference is again not significant, but similar results have been observed in most of the previous experiments which have continued for more than a day, and it may be that, as Freitag (1936) found for *Eutettix tenellus*, the capacity of the aphides for causing infection diminishes with increasing age of the individuals. In a short-lived insect such as *Myzus persicae* 2 or 3 days represents a considerable period of ageing.

(3) *Successive infections on three or four plants with constant infection feeding, and varying time on the healthy plants*

The last in the series of experiments with successive healthy plants (table 6) is given in rather greater detail than the others, for the results of each repetition of the experiment are presented instead of merely the totals for all repetitions. This is not because the results are of greater importance, but because: (*a*) the treatment on the different occasions was not identical, the series being carried on to the fourth plant only on the last three occasions; (*b*) the number of trials completed varied on the different occasions as not all the aphides completed the whole series of transfers; (*c*) the results varied considerably from occasion to occasion.

The irregular behaviour of the aphides on the different occasions may be accounted for by the fact that the experiment was carried out with the mild Wageningen strain of the virus during the winter months when development of symptoms is slow and erratic. The infectivity of the aphides was the same on the second plants as on the first, although the time spent on the first plants was 3 hr. In table 4 there was considerable reduction in infectivity after 4 hr. feeding on the first healthy plant, and there is no evidence that a large increase would result from a difference of only 1 hr. in the feeding times. Also from the results given in table 4 one would not expect an increase in infectivity even for 18 hr. feeding on the third healthy plant after a total of 6 hr. on the previous two. This increase is not in fact significant according to analysis of variance on the percentage infections, and it is obviously variable in occurrence and in magnitude. However, there does not appear to be reduction in infectivity such as is shown in table 4, and it might be suggested that the duration of infectivity, and possibly the period of delayed infectivity, vary with external conditions. If so, they appear to be lengthened by low temperatures or decreased daylight.

TABLE 6. CONSECUTIVE INFECTION OBTAINED ON SETS OF THREE OR FOUR HEALTHY PLANTS, AFTER 24 HR. FEEDING ON THE INFECTED PLANTS

	Infections obtained on			
Number of plants	First plant (3 hr.)	Second plant (3 hr.)	Third plant (18 hr.)	Fourth plant (3 hr.)
12	5	4	7	—
17	6	4	13	—
15	1	1	7	—
20	13	14	15	—
Total 64	25	23	42	—
19	7	14	16	4
19	11	15	11	6
18	7	5	9	2
Total 56	25	34	36	12
Percentage infection	42	48	65	21

Three hours feeding on the second day (fourth plant) gave fewer infections than corresponding periods on the first day (first and second plants).

This agrees with the results obtained in both previous experiments, and the reduction seems to be about the same whether the aphid was fed for the previous 24 hr. on one, two, or three, healthy plants (compare tables 4 and 5). The mere fact of transference from plant to plant, therefore, is not able to account for decrease in infectivity, but only the time actually spent in feeding.

(4) *Distribution of infection between the successive healthy plants*

In all the experiments which were made on successive infections the performance of each individual group was recorded separately, and the results can be arranged to show the groups which infected each plant in relation to their performance on the preceding and succeeding plants. To do this the percentage of aphides which infected the later plants is calculated according to whether they did or did not infect previous plants. For example, if out of 100 pairs of successive healthy plants fifty of the first plants and twenty-five of the second plants were infected, and if it is found that twenty of these twenty-five aphis groups, or 80 % of the second infections, belonged to the fifty which caused previous infection, and only five, or 20 %, did not cause previous infection, then the aphis community is composed of individuals of varying efficiency, the more efficient vectors causing most of the infections on both plants. If, on the other hand, the higher percentage of successes on the second plants occurs after failure on the first, this would indicate that the general level of efficiency is uniformly low, but that a relatively high percentage of insects do not develop infectivity until some time after ceasing to feed on the infected plants. Their virus might then be available either for the first plant or for the second, but generally insufficient for both.

Table 7 gives the percentages of aphides which infected successive plants after having failed or succeeded on the previous plants. The percentages are calculated mainly from experiments whose results have already been given in tables 4–6.

With one exception the highest percentage of infections on second, third or fourth plants were obtained by aphides which had already caused infection. When the interval between the successive plants was as much as 24 hr. all the aphides which infected the final plants had given an almost complete series of consecutive infections. Thus the only most efficient vectors retained their infectivity for very long periods, and these appear to have acquired sufficient virus to infect both early and late plants. Conversely, the aphides which hold up their infectivity for very long periods are weakly infective. Although their infectivity develops late it is less permanent than that of insects which become infective at an earlier stage.

Table 7. Distribution of infections among successive healthy plants. Data from tables 4–6 and from one other experiment

Time on infected plants hr.	Performance at previous trial	Percentage giving infection at subsequent trials		
(from table 4)		First: 1 hr. Second: 20 hr.	First: 4 hr. Second: 20 hr.	First: 20 hr. Second: 20 hr.
½	+	0	0	12
	0	2	2	0
2	+	66	58	3
	0	28	4	6
6	+	69	54	26
	0	52	13	0
18	+	77	64	20
	0	68	38	12
24	+	84	53	20
	0	44	12	0
(from table 5)		First: 24 hr. Second: 24 hr.	First: 24 hr. Third: 24 hr.	Second: 24 hr. Third: 24 hr.
24	+	21	0	0
	0	12	0	0
24 and 48	+	44	4	8
	0	11	0	0
(from table 6)		First: 3 hr. Second: 3 hr.	First: 3 hr. Third: 20 hr.	Second: 3 hr. Third: 20 hr.
18	+	66	70	77
	0	37	59	52
		First: 3 hr. Fourth: 3 hr.	Second: 3 hr. Fourth: 3 hr.	Third: 20 hr. Fourth: 3 hr.
18	+	31	33	13
	0	13	5	5
(unpublished data)		First: 2 hr. Second: 22 hr.	First: 2 hr. Third: 24 hr.	Second: 22 hr. Third: 24 hr.
4	+	70	23	20
	0	62	14	12

The exception mentioned above is from table 4, and concerns the aphides which were only given ½ hr. on the infected plants. Very few of these aphides developed any infectivity and then, with one exception, after failure on the first healthy plants, indicating that the virus charge of the aphides was only sufficient to infect the later plants if it had not been used up on the earlier ones. The aphis group which gave the single infection on a second plant after 20 hr. feeding on the first plant had also infected the first plant. This was probably caused by two aphides becoming infected in the same group, one later than the other, or by one aphis developing infectivity sufficient for two plants, just before its transfer to the second healthy plant. This single infection of a second plant appears in the table as 12% of successive infections (line 1, table 7), but this figure is obviously subject to large errors because of the small number of infections obtained on the first plants, viz. 8.

Even one second infection after 20 hr. feeding on the first plant is a higher proportion of second infections than would be expected from the results with aphides which had longer infection feeding times. It is probably accounted for by the suggestion made previously, that a larger proportion of weakly infective aphides than of highly infective ones tend to hold up their virus, i.e. there are proportionately fewer weakly infective aphides among the long infection feedings than the shorter ones.

The author has much pleasure in acknowledging the help of Miss F. M. Roberts, Ph.D., in carrying out these experiments.

SUMMARY

The efficiency of the vector *Myzus persicae* in transmitting sugar-beet yellows virus increased greatly with increasing feeding time on the infected plants. Infection was produced on a succession of healthy plants for 1, 2, or 3 days depending on the length of the infection feeding time. The infectivity of the vectors increased with increasing feeding time on the healthy plants undergoing infection, and decreased with increasing feeding time on healthy plants prior to those on which the infection trial was made. There was no clearly defined "incubation period" of the virus in the vector, below which no insect could cause infection, but there was variation in the time between cessation of infection feeding of the aphis and the initiation of infection in the healthy plants.

The relation of this virus with its vector differ from those of the viruses already described as *non-persistent* (Watson and Roberts 1939). For the latter viruses infectivity is lost by *M. persicae* soon after cessation of infection feeding; after fasting the vectors become optimally infective almost immediately on penetrating infected tissues of the leaf. Their infectivity decreases with increasing feeding time on the infected plants,

and increases only slightly with increasing feeding time on the healthy plants.

The behaviour of sugar-beet yellows virus is compared with that of curly-top virus of sugar-beet, in which infectivity also persists for an indefinite period in the vector and increases with increasing feeding time on infected and healthy plants.

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Ocular interaction in its relation to measurements of brightness threshold

By B. H. Crawford, M.Sc.

(*Communicated by Sir John Parsons, F.R.S.—Received* 11 *March* 1940)

1. Introduction

The question to which an answer is here sought is how far, if at all, the sensations from the two eyes of a subject add together in the measurement of brightness threshold. That is, do two eyes see better than one, or only equally well? Two aspects of the question have been investigated; first, the measurement of brightness threshold when the eyes are in equilibrium with a visual field of fixed brightness (steady state of adaptation); secondly, the measurement of the variation of brightness threshold with time after cutting off a conditioning field of more or less high brightness (changing state of adaptation). In an investigation of this kind the action of the eye pupils must be eliminated. This was done by the use of Maxwellian view for all visual fields, though the method of fixing the eye pupil at its maximum size by a mydriatic could also be used (Lythgoe and Phillips 1938). It was felt, however, that the Maxwellian view method was more generally satisfactory, though it necessitated more complicated apparatus and great care in equalizing the beams entering the two eyes.

Consideration is also given to the effect of a light stimulus applied to one eye on threshold measurements made with the other eye.

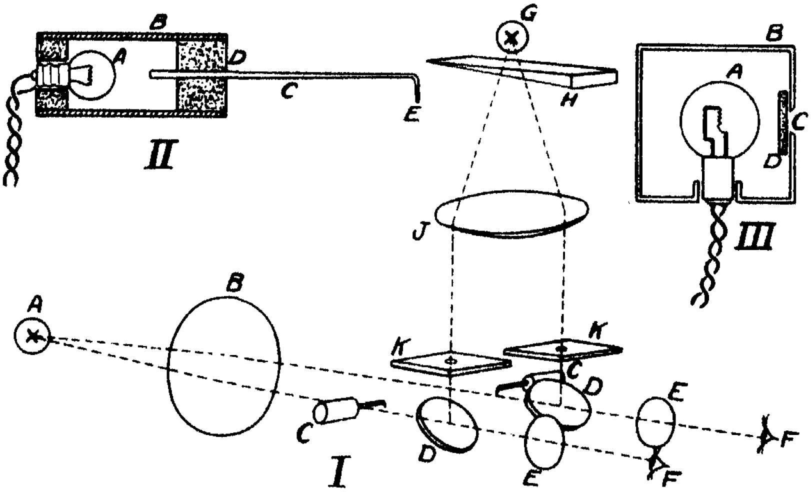

Figure 1. I. *Perspective view of binocular apparatus.* A, light source for illuminating conditioning fields (see III); B, large lens giving parallel beam from A; C, C, fixation points (see II); D, D, slightly wedge-shaped, plain glass mirrors; E, E, field lenses converging beams to focus in planes of eye pupils (F, F) of the subject; G, light source for illuminating test fields (see III); H, neutral wedge; J, large lens giving parallel beam from G; K, K, diaphragms to limit test field sizes. II. *Detail of fixation spots.* A, small lamp; B, tubular brass case; C, thin glass rod drawn down to point at E and mounted through cork D. III. *Detail of light sources.* A, lamp; B, brass case; C, aperture; D, opal glass.

2. APPARATUS

This is shown diagrammatically in figure 1, the general scheme of the apparatus at I, and the detailed construction of the light sources for conditioning and test fields, also of the fixation points, at II and III. The most important point about the apparatus is that the fields presented to the two eyes should be perfectly symmetrical in every way. Spatial symmetry was attained by mounting the field lenses, E, E, and the test field apertures, K, K, in graduated, mechanical microscope stages, so that their positions could be accurately adjusted; this allowed also for the final positioning of the exit pupils of the apparatus so that they fell centrally in the eye pupils of the subject, whose head was firmly fixed relative to the apparatus by biting on a sealing wax mouth grip. The fixation points, C, C, could be adjusted with sufficient accuracy by miniature holders of the retort stand type. This spatial symmetry, combined with the use of a single light source for both conditioning fields and another for the two test fields, led to satisfactory symmetry of brightness distribution; the differences between the fields for right and left eyes were in no case over 2 % and usually much less. Neutral filters and wedge for intensity regulation were put as close as possible to the light sources so as to preserve the symmetry of the apparatus.

The pattern of the visual field consists of a circular conditioning field of diameter subtending 16° at the eye, with a circular test field superposed on it at the centre. The size of the test field is specified for the various sets of data given later on.

3. RESULTS

3.1. *Steady state of adaptation*

In this section results are given for threshold measurements with the eyes in sensible equilibrium with conditioning fields of various brightnesses, using respectively both eyes, the right eye and the left eye. Foveal and parafoveal retinal areas were tested, using a test field of 0·67° diameter exposed for 1 sec. The results are given in table 1. The brightness units used here and later are candles per square foot seen through a pupil of 3 mm. diameter.

This table indicates that for the fovea at all brightness levels studied and for the parafovea at very low brightness levels there is only a small difference between the threshold sensitivity of monocular and binocular vision; it is probably due merely to the greater probability of seeing the test spot with two eyes than with one. For the parafovea at medium and high brightness levels, however, there is a significant difference in sensitivity; on the average, two eyes are about 30 % more sensitive than one eye to a difference in brightness.

A number of workers have from time to time been interested in this question of relative performance with monocular and binocular vision (Broca 1894; Stigler 1909; Krüss 1910; Lythgoe and Phillips 1938). Additional references are given by Lythgoe and Phillips. In all cases binocular vision has been found advantageous, but it is not always possible to deduce the magnitude of the advantage from the data given. It is probable that the advantage found in practical photometry is only partly due to an actual decrease in brightness difference threshold, which, as shown above, is small. The greater freedom from fatigue in binocular vision is likely to be quite as important in determining the precision of photometric readings. In the present work every precaution was taken to eliminate any purely fatigue effects, hence, perhaps, the relatively small difference between monocular and binocular sensitivity.

TABLE 1

Conditioning field brightness c./ft.^{2}	Threshold brightness (c./ft.^{2})			Ratio mean monocular to binocular threshold
	Both eyes	Left eye	Right eye	
		(*a*) Fovea		
0	0·00060	0·00066	0·00063	1·07
0·74	0·0148	0·0151	0·0186	1·13
51	0·89	0·91	0·93	1·03
		(*b*) Parafovea 8°		
0	0·0000141	0·0000155	0·0000151	1·09
0·0000040	0·0000141	0·0000155	0·0000155	1·10
0·000059	0·000071	0·000076	0·000079	1·08
0·00060	0·000270	0·000345	0·000370	1·32
0·0062	0·00093	0·00115	0·00132	1·32
1·10	0·044	0·054	0·051	1·18
12·9	0·76	1·07	1·05	1·39
48	2·95	4·25	3·15	1·24

3.2. *Changing state of adaptation: variation of threshold after cutting off conditioning field*

The conditioning field, of brightness 132 c./ft.^{2}, was observed for some minutes with both eyes, the left eye or the right eye; it was then cut off and threshold measurements made at intervals. For the early readings the time of appearance of a test field of fixed intensity was measured, with a new conditioning exposure for each test field intensity. The methods of taking these readings have been described in detail in a previous paper (Crawford 1937). Measurements were made for both foveal and parafoveal retinal areas, with a test field of 0·67° diameter. The results are shown in figure 2, in which each curve is the mean of three or four separate sets of measurements. There is a very definite difference between the monocular and binocular recovery curves for shorter times for both foveal and parafoveal vision, and a small, though still definite, difference at longer times.

It is difficult to postulate a mechanism to explain these differences, though a certain complexity is not unexpected, as some form of nervous interaction must take place eventually at higher centres on the way to, or in, the brain. The most concrete suggestion is that sponsored by Lythgoe and Phillips, namely, that in binocular vision twice the area of retina is being stimulated by the test spot, and this is equivalent to a test spot of twice the area seen by one eye only. The present work lends some support to this hypothesis, and the following summary table may be compared with the results of Lythgoe and Phillips.

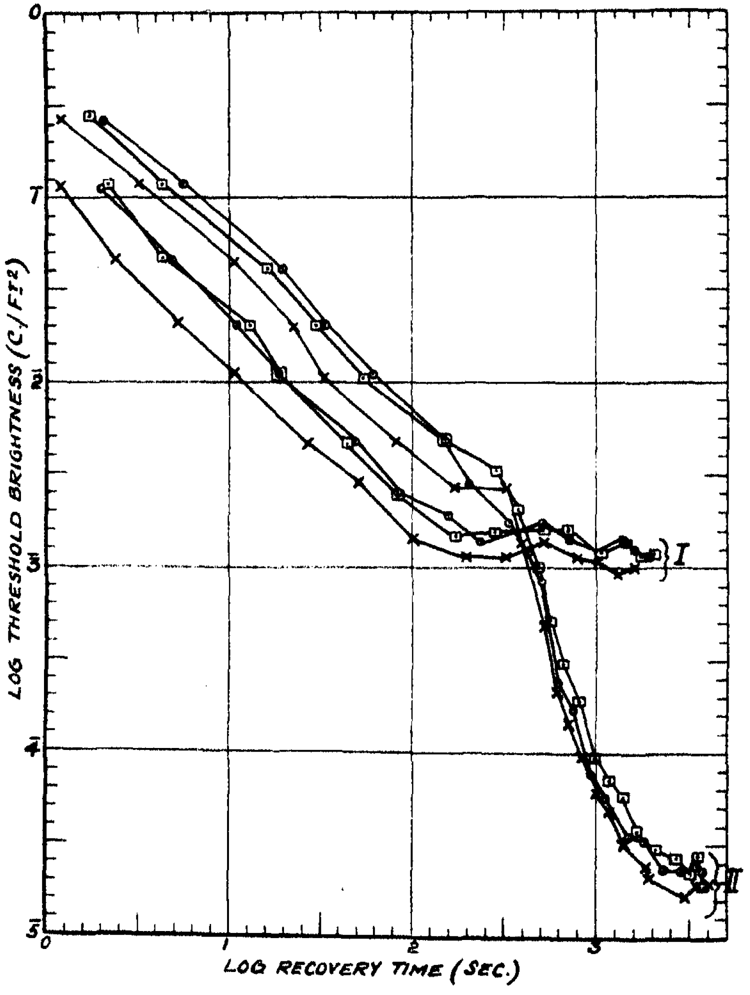

Figure 2. Subject B. H. C. Set I, fovea. Set II, parafovea 8°. Conditioning field brightness = 132 c./ft.², reduced to zero at time zero. Test field diameter, 0·67°, exposure 1 sec. ×, both eyes; ⊙, left eye; ⊡, right eye.

On the other hand, the results of § 3.1, for measurements under static conditions, seem quite at variance with this hypothesis, for under the very conditions where integration of visual effect might be expected to occur, namely, in parafoveal vision at low brightnesses, very little integration appears. A greater degree of integration appears, indeed, at higher brightness levels. One feels that the complete answer to this question has not yet been found.

TABLE 2

Time sec.	Ratio of mean monocular to binocular threshold	
	Fovea	Parafovea 8°
3	2·0	1·4
10	2·0	1·4
30	1·7	1·8
100	1·6	1·6
300	1·3	0·85
1000	1·1	1·3
3000	—	1·3

From the point of view of carrying out measurements on visual properties, such as a more detailed investigation of the course of dark adaptation, it is useful to note that the shapes of the curves of figure 2 are only slightly modified by the use of monocular instead of binocular vision; there is no fundamental alteration in shape, so that conclusions drawn from experiments employing monocular vision will probably not be invalidated thereby. This is a point of some practical importance, as complete binocular apparatus for Maxwellian view of all visual fields is complicated, and it is generally desirable to use monocular apparatus if suitable.

4. INFLUENCE OF STATE OF ADAPTATION OF ONE EYE ON THE THRESHOLD SENSITIVITY OF THE OTHER EYE

It appears from the work of anatomists that there exist certain more or less direct nervous connexions between two eyes, also that optic nerve fibres from one eye go to the receptive area of the brain which is mainly concerned with the other eye. Thus it is possible that a light stimulus falling on one eye might affect brightness threshold measurements made with the other eye. Several workers have obtained experimental data on this point with divergent results (Shearer 1932; Kravkov 1934, 1937), or, at all events, divergent opinions about their results. Shearer finds no interaction between the two eyes, Kravkov some degree of interaction under certain conditions.

The binocular threshold apparatus described above is obviously at once adaptable for investigation of this question, and the following table is typical of the results obtained. One eye was exposed to conditioning fields of various brightnesses, while the other eye made brightness threshold measurements against a field of zero brightness.

TABLE 3. PARAFOVEA 8°. RIGHT EYE EXPOSED TO CONDITIONING FIELD. LEFT EYE EXPOSED TO TEST FIELD (DIAMETER 0·67°)

Conditioning field brightness (c./ft.^{2})	Threshold brightness c./ft.^{2}
0	0·0000174
0·0000040	0·0000209
0·000059	0·0000234
0·00060	0·0000159
0·0062	0·0000135
0·093	0·0000186
1·26	0·0000186
12·9	0·0000141
132	0·0000178

From this table it is plain that there is no detectable interaction between the two eyes, at least up to the highest level of field brightness used, in agreement with Shearer. The interaction found by Kravkov occurred when the threshold measurements were made against a field not of zero brightness. This condition is not considered here, but it is possible that in Kravkov's work some stray light entered the eye which was making threshold measurements. Even with the most careful screening, light might be scattered from one eye to the other through the frontal head tissues, unless Maxwellian view of the visual fields was employed.

From a practical point of view the above results are useful, as they demonstrate that in taking monocular threshold readings, especially at very low brightness levels, no particular care need be exercised in keeping the non-observing eye dark adapted or, indeed, in any particular state of adaptation before or during the readings.

Acknowledgements are due to the Illumination Research Committee, under whose auspices the above work was done.

5. SUMMARY

It is found that in all cases binocular vision produces a lower threshold than monocular vision, but, under static conditions of foveal vision at all brightness levels and of parafoveal vision at very low levels, the difference

is small and can be wholly explained by the fact that with two eyes there is a greater probability of the test field being seen than with one eye. For parafoveal vision under static conditions at medium and high brightnesses, binocular vision gives a threshold about 30 % lower than does monocular vision. When the state of adaptation is changing—after cutting off a conditioning field of high brightness—both foveal and parafoveal retinal areas show more rapid recovery for binocular than for monocular vision. In the fovea the difference is greater in the initial stages of recovery of dark adaptation, while in the parafovea the difference is approximately constant during the whole process.

It is also found that when one eye is used for making threshold measurements it is completely unaffected by exposure of the other eye to fields of brightness up to 130 c./ft.^{2}.

Any subsidiary pupil diameter effects are avoided by the use of Maxwellian view of the visual field.

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END OF THE ONE HUNDRED AND TWENTY-EIGHTH VOLUME (B)

ABSTRACTS

OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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12 JANUARY 1940

On the photon component of cosmic radiation and its absorption coefficient. By B. ROSSI and L. JÁNOSSY. (*Communicated by P. M. S. Blackett, F.R.S.—Received* 17 *November* 1939.)

Photons, unlike ionizing particles, cannot be observed directly with counters. Owing to this difficulty very few investigations dealing with the photon component of cosmic radiation have been carried out. In the present paper a simple method is described which is suitable for the observations of cosmic-ray photons. The transition effect of photon-initiated showers in lead has been measured. The result is in agreement with the predictions of the cascade theory of Bhabha and Heitler as extended by Arley. The absorption coefficients of cosmic-ray photons have been measured in lead iron and aluminium and they agree with the theoretical values as given by Bethe and Heitler. The lower limit for the total flux of cosmic-ray photons, above 10^7 eV, has been found to be 10 % of the ionizing component of the cosmic-ray beam.

The combustion of aromatic and alicyclic hydrocarbons. III. Ignition and cool-flame characteristics. By J. H. BURGOYNE, D. M. NEWITT and T. L. TANG. (*Communicated by A. C. Egerton, Sec.R.S.—Received* 22 *November* 1939.)

The general ignition characteristics of eleven aromatic and alicyclic hydrocarbons have been investigated, and previous conclusions regarding their relative reactivity to oxygen are confirmed.

While benzene, toluene, and ethylbenzene show smooth ignition curves within the limits of pressure available, the other hydrocarbons examined exhibit low-temperature ignition systems and, except in the case of *p*-xylene, cool flames are propagated.

Cyclohexane behaves as a typical paraffin, but tetrahydrobenzene, while resembling the olefines in most respects, has cool-flame limits characteristic of the aromatic series.

Chemical analyses of the ultimate products of slow combustion and cool-flame reactions show that the two types of combustion yield similar results at the same temperature. The low-temperature systems of the aromatic hydrocarbons are similar to those of the paraffins and what differences occur appear to be conditioned merely by the relative stability of analogous intermediate products.

The combustion of aromatic and alicyclic hydrocarbons. IV. The kinetics of the slow combustion of benzene and its mono-alkyl derivatives at low temperatures.

By J. H. Burgoyne, Ph.D. (*Communicated by A. C. Egerton, Sec.R.S.—Received* 22 *November* 1939.)

The kinetics of the combustion of benzene and the four simplest mono-alkyl derivatives have been studied in the temperature range between 250 and 400° C, and the results compared with those previously obtained relating to the region above 400° C.

It is concluded that, excepting in the case of benzene itself, the oxidation of these fuels consists of two co-existent chain reactions, as is thought to be the case with the aliphatic hydrocarbons also. Of these processes, one is predominant below 300° C and the other above 400° C, whilst in the intervening range of temperature the two mechanisms proceed on terms of approximate equality. The low-temperature reaction on intensification gives rise to cool flames, while from the high-temperature mechanism a chain-thermal ignition results. The kinetic characteristics of the two processes are demonstrated and compared.

In the intermediate temperature zone (300–400° C), the prominence of the process of cool-flame initiation is thought to be the partial cause of the observed increase in the "order of reaction", and it is suggested that the negative temperature coefficient is due to the mutual interaction of the co-existent chain reactions.

The equilibrium and lattice-spacing relations in the system magnesium-cadmium.

By W. Hume-Rothery, F.R.S. and G. V. Raynor. (*Received* 23 *November* 1939.)

The equilibrium diagram of the system magnesium-cadmium has been investigated by thermal, microscopic, and X-ray methods. The liquidus and solidus curves pass smoothly from the melting point of magnesium to that of cadmium, with no indication of a discontinuity characteristic of a peritectic reaction. Microscopic methods show no indication of a two-phase region above the temperatures of the superlattice transformations. The supposed compound $MgCd_2$ of Hume-Rothery and Rowell is not a true constituent of the system magnesium-cadmium, but is an oxide-nitride complex formed when the alloys are exposed to the atmosphere. It is formed most readily when the composition of the alloy corresponds with the formula $MgCd_3$; a somewhat similar effect has been noted in the system nickel-chromium. The two-phase region claimed by Grube and Schiedt is not confirmed.

The lattice spacings of a complete series of alloys at 310° C have been measured with a high-temperature X-ray camera. The first additions of cadmium to magnesium cause a contraction of the *a* parameter of the unit cell, but have little effect on the axial ratio, so that the *c* parameter contracts in almost the same proportion. With further additions of cadmium, the axial ratio increases slightly. The addition of magnesium to cadmium causes an expansion of the *a* parameter, but, at the same time, the axial ratio decreases so markedly that the *c* parameter diminishes, so that each metal decreases the *c* parameter of the other. A stage is then reached at which the solid solutions derived from magnesium and cadmium acquire identical lattice spacings, which persist unchanged over a narrow range of composition. These effects are discussed, and are shown to be in agreement with the Brillouin Zone theory developed by Jones.

A current-meter for measuring turbulence. By A. T. DOODSON, F.R.S.

(*Received* 25 *November* 1939.)

The measurement of tidal currents presents many difficulties, and many types of apparatus have been designed with special objects in view. These types are reviewed and the advantages of instruments of universal type, capable of recording tidal currents moment by moment, are emphasized. Instruments for recording turbulence need to be very sensitive, yet robust, and the lack of suitable instruments led to the development of an electrically operated current meter which has been constructed with the aid of grants from the Government Grant Committee of the Royal Society. The apparatus is described in detail. It is highly sensitive to weak currents, has a scale practically linear with the velocity of current, and it gives records as frequently as at intervals of 15 sec. If required, velocities only can be registered continuously on a very open time scale. It has been used at sea for many weeks, and has given much satisfaction in performance.

The thermal decomposition of nitrogen iodide. By F. R. MELDRUM.

(*Communicated by W. E. Garner, F.R.S.—Received* 27 *November* 1939.)

The thermal decomposition of nitrogen iodide has been studied at low pressures and the reaction shown to be $2NH_3.NI_3 \rightarrow 2NH_3+N_2+3I_2$. The activation energy from the temperature coefficient and from calculations made with the aid of the Polanyi-Wigner equation is 18–19 kcal. The reaction is retarded by ammonia and water, and also by an increase in pressure over the solid.

The mechanism of the reaction is discussed in the light of the above results and of the calorimetric results quoted in the following paper.

The heat of formation of nitrogen iodide. By F. R. Meldrum. (*Communicated by W. E. Garner, F.R.S.—Received* 27 *November* 1939.)

From measurements of the heat of reaction of nitrogen iodide with excess of aqueous hydriodic acid, it is shown that the heat of formation of nitrogen iodide from the elements is -34 kcal./mol.

The reciprocity failure of photographic materials at short exposure times. By W. F. Berg. (*Communicated by C. E. K. Mees, F.R.S.—Received* 28 *November* 1939.)

According to recent conceptions on the formation of the photographic latent image, electric and ionic movements occur side by side. For sufficiently short times of exposure, the electronic process should be over before the less mobile ions move appreciably. Thus, for a range of short exposure times up to a certain maximum, the latent image should be formed in essentially the same manner. Electronic and ionic processes are then completely separated, and reciprocity failure, which is explained by the simultaneous occurrence of these two processes, should disappear. This is found to be the case, and the maximum time at which reciprocity failure begins to occur is found to be of the order of 4×10^{-5} sec. at room temperature for four different emulsions, and irrespective of the density. This maximum time can be calculated from data on ionic conductivity and agrees reasonably well with the above figure and also with the variation of the maximum with temperature. This is in agreement with the earlier result that at very low temperatures reciprocity failure was absent over the whole range of exposure times investigated.

The effect of unilateral castration on the production of spermatozoa. By Joseph Edwards. (*Communicated by J. Hammond, F.R.S.—Received* 29 *November* 1939.)

The effect of unilateral castration in the sexually mature rabbit on the production of spermatozoa has been studied quantitatively by means of semen exhaustion tests.

The frequency of collection is shown to have kept pace with the rate of production of spermatozoa by the testis. Contrary to what occurs in the ovary, there is no hypertrophy of spermatogenic function or of weight of testis following unilateral castration. There is a highly significant positive relationship between the weight of the testicular substance and the numbers of spermatozoa which it produces. The passage of spermatozoa through the epididymis may be accomplished in 4–7 days.

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1 FEBRUARY 1940

The measurement of tides by means of the Favé pressure gauge. By R. H. CORKAN, A. T. DOODSON, F.R.S., and J. PROUDMAN, F.R.S. (*Received* 13 *December* 1939.)

The measurements and analyses described in this paper were made with the object of calculating the variable parts of the components of the horizontal gradients of pressure in the sea. For such a purpose, and in the part of the Irish Sea considered, the principal harmonic constituent of the tidal elevation must have its amplitude determined to the nearest cm. and its phase determined to the nearest 0·3°.

The instruments used were three models of the *marégraphe plongeur* Favé. Though this instrument has been described in great detail by its designer, very little has been previously published on results obtained with it. Our greatest difficulties were in connexion with the calibration of the instruments.

The results obtained from the records taken at sea may be regarded as giving a good determination of the principal harmonic constituent of the tides at the stations considered, but not sufficiently good for a reliable calculation of the horizontal pressure gradients.

The investigation has given us very valuable experience, and when opportunity next occurs we intend to make further measurements.

The dynamic constants of human muscle. By A. V. HILL, SEC.R.S. (*Received* 14 *December* 1939.)

The characteristic equation of muscle is modified to meet the case of a constant mass accelerated instead of a constant force overcome at uniform speed.

The theory is compared with experimental results obtained on human arm muscles with an inertia wheel. The agreement is good.

The value of the dynamic constant b is deduced. Its order of size is muscle length per second. P_0/a cannot be very far from 4, its mean value in frogs' muscle.

The maximum speed of shortening (unloaded) is determined experimentally. This is of the order of size P_0b/a deduced from theory.

The mechanical efficiency, the optimum speed, the possibility of reflex inhibition during rapid shortening, certain practical applications and some proposals for further research are discussed.

The photolysis of ammonia. By E. A. B. Birse and H. W. Melville. (*Communicated by J. Kendall, F.R.S.—Received* 15 *December* 1939.)

The anomalously low stationary hydrogen atom concentration during the photodecomposition of ammonia is due either to the fact that the primary photochemical efficiency is not unity or that the hydrogen atoms are removed at an abnormally fast rate. It is shown by utilizing the para-hydrogen conversion that photodecomposing ammonia has no effect on the stationary hydrogen atom concentration of the conversion and therefore the low concentration of the ammonia photolysis must be due to inefficiency of the primary process. The primary efficiency is determined by a careful comparison of the mercury sensitized and the ammonia sensitized conversion of para-hydrogen. A method is developed for measuring accurately the rate of production of hydrogen atoms, which method is essential to the foregoing determination. This primary efficiency is found to be 0·58.

A method is also developed for measuring the lifetime of amine radicals.

Similar experiments with trideuteroammonia are also described. The primary efficiency is 0·28.

A revised and simplified scheme for the ammonia photolysis is given as a result of these new experiments.

The reaction of atomic hydrogen with hydrazine. By E. A. B. Birse and H. W. Melville. (*Communicated by J. Kendall, F.R.S.—Received* 15 *December* 1939.)

The efficiency of the reaction of atomic hydrogen with hydrazine has been measured in the temperature range 20–200° C by comparison with that of the hydrogen atom sensitized conversion of para-hydrogen. A static system is employed, the hydrogen atoms being produced by excited mercury atoms. The energy of activation is nearly the same as that of the para-hydrogen conversion, namely *ca.* 7 kcal. and the steric factor is *ca.* 10^{-2}. From these results it is shown that hydrazine is not responsible for the low stationary hydrogen atom concentration in the photolysis of ammonia as has hitherto been supposed.

Although the hydrogen atoms react with hydrazine very efficiently the quantum efficiency of this reaction is only 0·4 instead of the expected value of 2. An explanation of this result is suggested.

It is also shown that photodecomposing hydrazine converts para-hydrogen. This is taken to mean that the primary decomposition is represented by

$$N_2H_4 + h\nu = N_2H_3 + H.$$

The quantum efficiency of the mercury sensitized decomposition of hydrazine has been found to be 0·37. An explanation of this unexpectedly low value is given.

A new simplified method of analysing the ternary mixture p-H_2—n-H_2—N_2 *is* described.

The phosphorescence of various solids. By J. T. RANDALL and M. H. F. WILKINS. (*Communicated by M. L. E. Oliphant, F.R.S.—Received* 18 *December* 1939.)

A complete understanding of luminescence phenomena in solids involves the study of such properties as emission and absorption spectra, decay laws of phosphorescence, and photoconductivity. In this paper accurate results on the decay laws of phosphorescence of a number of solids are given together with corresponding observations on photoconductivity. Measurements of the phosphorescence of pure and impure solids have been carried out by two methods. In the first and more accurate method the intensity of phosphorescence of an annulus of powder on a rotating disk was measured by means of a vacuum photocell and d.c. amplifier of the electrometer triode type. In the second method an intermittent beam of ultra-violet radiation falls on a stationary specimen; during the illumination period the intensity of luminescence rises to an equilibrium value, afterwards decaying when the ultra-violet radiation is cut off. By allowing the varying luminescence radiation to fall on the primary cathode of an electron multiplier coupled with an oscillograph, rise and decay curves of various solids can be obtained on the oscillograph screen and photographed for measurement.

In general the results are not simple for compounds activated with impurity. Pure uranyl salts, however, give a simple exponential decay $I = I_0 e^{-\alpha t}$; moreover, the salts are not photoconductors. The results confirm the idea that the luminescence of these salts is a property of excitation states within the co-ordination group of the uranyl ion. The time of decay suggests that forbidden transitions are involved. Tungstates on the other hand are slightly photoconducting in the pure state, and have a very fast decay.

A number of inorganic solids containing manganese impurity have also been examined; these include cadmium chlorophosphate, zinc mesodisilicate, cadmium silicate, cadmium borate, zinc beryllium silicates and zinc orthosilicate. In all these solids the initial decay is approximately exponential with a slight curvature of the semi-log plot convex with respect to the time axis. In the zinc beryllium silicates and in zinc orthosilicate this curvature is more pronounced, and in the latter case develops into a long phosphorescent tail. In all cases, however, the slope is a function of time and not of phosphorescence intensity, thus showing that the decays are not of the bimolecular type. The photoconduction results show that the pure non-fluorescent compounds and the impure fluorescent ones conduct in general to the same extent. We are inclined to the view that the photoconduction arises from secondary causes such as lattice defects or interstitial matrix atoms. In the solids cadmium chlorophosphate, zinc mesodisilicate, cadmium silicate, and cadmium borate, we suggest that the decay law is of the type $I = \Sigma I_0 e^{-\alpha t}$; such a result can easily arise from the non-equivalent positions of impurity atoms in various parts of the crystal. From the slope of the semi-log plot the lifetime of the excited manganese ion may be calculated.

The long tail observed for most zinc orthosilicates can best be explained by supposing that the absorption process first sets free the excited electrons. This is followed by electron trapping probably in the neighbourhood of the manganese ions. The free electrons recombine with manganese ions very rapidly; these electrons, however, remain in the excited state an appreciable time, thus giving rise to the initial exponential decay. The phosphorescent tail depends only on the rate at which electrons are released thermally from trapped positions.

Comparison of the acceleration due to gravity at the National Physical Laboratory, Teddington, and the Bureau of Standards, Washington.

By B. C. Browne and E. C. Bullard. (*Communicated by Sir Gerald Lenox-Conyngham, F.R.S.—Received* 20 *December* 1939.)

The values of the acceleration due to gravity at the points used for the recent absolute determinations at the National Physical Laboratory, Teddington, and at the National Bureau of Standards, Washington, D.C., have been compared. The difference found is $1\cdot096_9$ cm./sec.^{2}. The difference between the two absolute determinations is $1\cdot101_5$ cm./sec.^{2}, whilst that deduced from previous indirect relative connexions is $1\cdot095_3$ cm./sec.^{2}. The agreement is within the uncertainties of the measurements, and leaves little doubt that the hitherto accepted value at Potsdam is some seventeen parts in a million too great.

The kinetics of decarboxylation in solution.

By P. Johnson and E. A. Moelwyn-Hughes. (*Communicated by R. G. W. Norrish, F.R.S.—Received* 21 *December* 1939.)

The kinetics of the decarboxylation of trichloracetic tribromacetic, and trinitrobenzoic acids have been studied over extended temperature ranges in aqueous solution. The results conflict with the formula of Arrhenius, and conform better to an equation of the type

$$\ln k = c + \frac{J}{R} \ln T - \frac{E}{RT}.$$

The experimental values of J are found to be negative. Decarboxylation reactions thus come into line with catalysed and uncatalysed hydrolyses in aqueous solution.

Certain theoretical considerations are advanced to account for the observations.

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8 MARCH 1940

Crystal boundaries in tin. By B. CHALMERS. (*Communicated by E. N. da C. Andrade, F.R.S.—Received* 2 *December* 1939.)

A new technique is described for preparing specimens of tin consisting of two crystals of controlled orientation, of such a size and shape that a tension can be applied transversely to the boundary. It is found that the temperature at which the crystals separate under tension is below the melting point of the crystalline material, and that the difference is independent of the angle between the crystal axes of the two crystals and of impurity providing this does not exceed about 0·02 %. The mechanism of boundary formation is considered, a distinction being drawn between two types of boundary described as "columnar" and "segregational". The structure of the boundary is discussed in the light of the new experimental evidence, which favours a "transitional lattice" rather than an "amorphous layer" theory.

The paedogenetic male cycle in *Salmo salar* L. By J. W. JONES and J. H. ORTON. (*Communicated by C. T. Regan, F.R.S.—Received* 11 *December* 1939.)

In a further study of the male sex phases throughout the year in salmon parr—accompanied by histological investigation of the gonad—the earlier observations have been confirmed. Some 2790 parr and smolts, captured mainly by rod and line from English and Welsh rivers, have been examined; 50 % of these were male. All the females are immature virgins; the males exhibit a seasonal sexual cycle in which the gonad develops in the summer, becomes mature in the autumn and is spent in winter. After spawning, a high proportion of the male parr migrate to sea as smolts. All the male smolts examined, i.e. 490, had quiescent testes, being either spent or immature and virgin. It is hereby established that two types of male smolts migrate to sea. The gradual disappearance of ripe male parr during the spawning season (beginning about November) and the correlated appearance and increase of males with spent gonads renders it virtually certain that the ripe male parr spawn on the salmon redds.

The temperature ranges in the rivers during the development of the male gonad is noted.

The finding of only 60 % spent amongst the migrating smolt males as against 75 % ripe spending and spent male parr in the preceding spawning period, November and December, may be an indication of a slight mortality among the spawning males.

The sex records given herein, along with those of ripe parr in the literature, leave no doubt that paedogenesis in the male of the Atlantic salmon occurs throughout its geographical range.

The remarkable paucity of males amongst the younger classes of immigrating salmon noted by Hutton on the Wye may be general and be compensated biologically by the abundant paedogenetic males.

Histological changes in the testis in the sexual cycle of male salmon parr.

By J. W. Jones. *(Communicated by C. T. Regan, F.R.S.—Received* 11 *December* 1939.)

Over 2500 salmon parr and smolts have been examined for condition of the gonad. The male gonads were classified according to the nomenclature of the International Council. All female gonads were immature, and the development of the male gonad was found to be seasonal. Spermatogenetic development begins from late May to July, and ripe sperm was first noticed in late September. The greatest percentage of ripe testes was taken in late October and November (the adult spawning time), during which period ripe sperm was extruded on the application of slight pressure to the abdomen. From November–December to June–July spent testes in various stages were found.

The ripening and spending of the testes may be followed macroscopically. The youngest testes sampled are paired thread-like bodies extending the length of the body cavity. As development proceeds the testes widen anteriorly; such widening extends backwards with increase in size. The ripe testes occupy almost all available space in the body cavity. The rosy pink colour of the thread-like immature testes changes to the greasy white of the almost ripe condition (stages IV and V), and eventually the milky white of a ripe testis.

As spawning proceeds a general collapse of the testes takes place, accompanied by a correlated series of colour changes. When almost all the sperm had been extruded the testes were similar in size to the II and II+ developmental stages, but were of a peachy pink colour with white spots of relict sperm, and often with much relict sperm in the wide collapsed ducts. In the last stage in spending which could be ascertained macroscopically the testis was of a fawny colour with faint longitudinal striae, and had a rather wide duct.

Spermatogenic changes in the tube-like stage I were confined to an inward migration of the germ cells and their transformation into germ mother cells. These cells are large with lightly staining nuclei and cytoplasm and one or two plasmosomes; in the I+ stage they are more numerous and begin producing occasional spermatogonia. Further increase in size of the gonads is correlated with an increase in the number of the later spermatogenic products. Sperm appears in the larger II+ stages and is fairly abundant in the stage III gonads. Sperm increased in number at the expense of the earlier germ cells. In the almost ripe stage V testis and the ripe stage VI the testicular crypts are full of sperm with a few spermatids: secondary spermatocytes occur in the very tenuous crypt walls.

With the collapse of the testes due to extrusion of the sperm the outer testicular tunica becomes thicker and folded, the crypt walls become more pronounced, and the crypt cavity decreases in size. Germ mother cells reappear in the emptied testes; it is thus possible that the testes may develop more than once in the river.

No actively developing testes were found in the migrating fish.

The dehydration of crystals of chrome alum. By J. A. Cooper and W. E. Garner, F.R.S. (*Received* 27 *December* 1939.)

Measurements have been made of the rate of growth of dehydration nuclei over the 111 surfaces of crystals of chrome alum for a range of temperatures. These rates have been compared with the rates of penetration into the crystal. The self-cooling of the crystals has been measured and the heat of dissociation calculated both from the self-cooling and the temperature coefficient of the dissociation pressure. The heat of dissociation, 10 kcal., is much less than the calculated activation energy for the dehydration, viz. 31 kcal. An explanation is advanced for the abnormally high activation energy.

It is shown that small pressures of water vapour cause an acceleration of the rate of dehydration.

Investigations of infra-red spectra. Determination of C—H frequencies (~3000 cm.$^{-1}$) in paraffins and olefins, with some observations on "polythenes". By J. J. Fox and A. E. Martin. (*Communicated by Sir Robert Robertson, F.R.S.—Received* 3 *January* 1940.)

The infra-red absorption spectra of some twenty hydrocarbons in carbon tetrachloride solution have been studied in the region 2·6–3·8 μ with the following results:

$=CH_2$ groups in ethylene and higher olefins give rise to two CH frequencies with mean values 3079 and 2978 cm.$^{-1}$, corresponding, respectively, to unsymmetrical and symmetrical valency vibrations of the CH_2 group.

$\gg\mathrm{CH}$ groups in olefins have usually a single CH valency frequency close to 3019 cm.$^{-1}$, i.e. almost half-way between the two bands of the $=\mathrm{CH}_2$ group. The $-\!\!\!>\!\mathrm{CH}$ frequency in saturated compounds is 2890 cm.$^{-1}$.

$>\mathrm{CH}_2$ groups have normally two CH valency vibrations with mean values 2926 and 2853 cm.$^{-1}$, corresponding, respectively, to the unsymmetrical and symmetrical vibrations within the CH_2 group. In long-chain paraffins the position of these bands and their intensity per CH_2 group remain remarkably constant from one compound to another. As found previously, bands additional to these two may occur in some cases.

CH_3 groups also have two main CH valency frequencies, corresponding to unsymmetrical and symmetrical vibrations within the CH_3 group. These frequencies are close to 2962 and 2872 cm.$^{-1}$, respectively, when the methyl group is attached to another carbon atom, but in methyl bromide the frequencies are 3049 and 2958 cm.$^{-1}$, i.e. 87 and 86 cm.$^{-1}$ higher. The CH force constant in methyl bromide is almost exactly the same as in methane, and this indicates that the CH internuclear distance does not differ from that in methane by as much as 0·001 A. The CH force constant in a CH_3 group attached to a carbon atom is nearly 6 % lower than in methane and methyl bromide. In saturated hydrocarbons the position of the methyl bands and their intensity per CH_3 group remain fairly constant, but when a methyl group is adjacent to a double bond, as in propylene, the positions and intensities of the bands vary greatly from one compound to another for comparatively small changes in the molecule. The band near 2872 cm.$^{-1}$ is split in these compounds, the mean position of the two components being close to 2872 cm.$^{-1}$. The total area of all the bands attributable to CH_3 groups in the unsaturated molecules, although somewhat lower than in saturated compounds, is still, however, approximately proportional to the number of CH_3 groups present.

Identification of the *cis* and *trans* forms of 2-butylene is readily carried out by examination of the infra-red spectra. A striking resemblance exists between the spectrum of the *trans* form and that of propylene.

Two samples of a polythene (condensed ethylene resinoid) have been examined and have spectra similar to those of long-chain normal paraffins. A band at 2960 cm.$^{-1}$ characteristic of the methyl group is found in all cases and the proportion varies from 1 CH_3 : 8 CH_2 for the most soluble part of the polythenes to 1 CH_3 : > 0 CH_2 for the whole sample. The significance of these results in relation to X-ray examination is briefly discussed.

CH force constants are given for eleven varieties of CH bond, and mean integrated absorption areas are given for $\gg\mathrm{CH}$, $-\!\!\!>\!\mathrm{CH}$, $=\mathrm{CH}_2$, $>\mathrm{CH}_2$ and CH_3 groups.

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28 March 1940

A study of the physical and chemical properties of the insect cuticle.

By G. Fraenkel and K. M. Rudall. (*Communicated by Sir William Bragg, P.R.S.—Received* 4 *January* 1940.)

The morphological and histological changes in the cuticle during the formation of the puparium in cyclorrhophous flies are described. The pupal contraction, resulting in the barrel shape of the puparium, is brought about by muscular action. If the central nervous system is separated from the muscles, a puparium is formed retaining the shape of the larval cuticle. During the conversion of the larval cuticle into the puparium the following physical and chemical processes take place:

(*a*) The arrangement of the chitin crystallites changes from random orientation in planes parallel to the surface to an arrangement in the white pupa cuticle and puparium where they lie approximately parallel to the transverse axis.

(*b*) In the larval and white pupa cuticles the chitin crystallites can be rotated in any direction by forces of compression or extension. This freedom of rotation is lost in the hard brown puparium.

(*c*) The swelling power of the puparium is very considerably less than that of larval or white pupa cuticles. In the puparium it is about the same as in purified chitin itself.

(*d*) The dry weight of the puparium increases to about 125 % of that of the larval cuticle.

(*e*) There is a marked decrease in the water content of the cuticle. This dehydration is regarded as having a chemical basis.

(*f*) The larval cuticle contains about 60 % chitin and the puparium about 47 % chitin. During the formation of the puparium the absolute amount of chitin remains unchanged.

It is considered that the whole of the non-chitinous substance in the larval cuticle consists of protein which is comparatively water-soluble in the larval cuticle and water-insoluble in the puparium. This protein, for which the name "insectin" is proposed, shows peculiar properties. It is characterized by high solubility in hot water and after being precipitated in trichloracetic acid it redissolves on heating.

The hardening of the puparium can be attributed to the following: the higher degree of chitin orientation; the closer packing by dehydration of the substances which form the puparium; the increasing insolubility of the protein; the loss of swelling power in the puparium; the change from a dispersed phase of chitin in protein to a condensed chitin-protein association. According to the X-ray evidence this seems to consist of a mixed crystallization of chitin and protein.

It is not necessary to postulate the existence of special incrustations, and the term "chitinization", hitherto often used for describing the process of hardening in the insect cuticle, is misleading.

Relaxation methods applied to engineering problems. VI. The natural frequencies of systems having restricted freedom. By A. Pellew and R. V. Southwell, F.R.S. (*Received* 12 *January* 1940.)

In this paper relaxation methods are applied to the difficult problem of determining the natural modes and frequencies of a system having restricted but considerable freedom (i.e. where the independent co-ordinates number six or more). A theorem published recently is used to impose lower limits on the wanted frequency, and upper limits are calculated on the basis of "Rayleigh's principle". In the "liquidation" process these upper and lower limits converge steadily, and when they have become closely coincident both the frequency and the associated mode (which "Rayleigh's principle" does not determine) are given with sufficient accuracy for all practical purposes.

An example treated in detail relates to undamped torsional vibrations or (by mathematical analogy) to transverse vibrations of a tensioned string. Concluding sections indicate some immediate extensions of the method, viz. (*a*) to vibrating systems which are entirely unconstrained, (*b*) to dissipative systems in which the damping forces (as is usual in practice) are relatively small, (*c*) to continuous systems, for which the method is an alternative to that given in Part IV of this series.

New termite intercastes. By A. M. Adamson. (*Communicated by A. D. Imms, F.R.S.—Received* 15 *January* 1940.)

An individual of *Nasutitermes* (*Nasutitermes*) *guayanae* (Holmgren) in the Termitidae, representing the only known termite intercaste combining intimately many important features of two castes, was found in Trinidad, B.W.I., in March 1937. The head is soldier-like in most features, but has compound eyes, ocelli and some other characters of the reproductive caste. The pronotum is intermediate between that of the soldier and the reproductive castes; the wing-buds are somewhat like those of a reproductive nymph, except that the pigmentation approaches that of an imago.

Two soldiers of *Microcerotermes arboreus* Emerson, also in the Termitidae, with wing-buds, abnormally large vestigial eyes, and with the pronotum intermediate between those of the soldier and reproductive castes, were found in an observation colony, in Trinidad, in June 1937.

Previously reported termite intercastes appear to be merely soldiers with wing-buds in a few species of the primitive Kalotermitidae and worker-soldier intercastes in *Reticulitermes* (Rhinotermitidae) and *Nasutitermes similis* Emerson and *N. myersi* Snyder (Termitidae).

The significance of termite intercastes is discussed. They support the view of Emerson and others that the worker caste arose phylogenetically from the soldier. An explanation of the origin of the intercastes, and their relation to theories of caste determination, must await further advances in the study of the latter problem.

The dielectric capacity of electrolytes in mixed solvents: ion association in solutions of magnesium sulphate.

By A. W. Mason and W. J. Shutt.

(*Communicated by W. C. M. Lewis, F.R.S.—Received* 16 *January* 1940.)

Experimental details are presented for an improved "force" method to be applied to the determination of the dielectric constants of conducting liquids. Particular attention is devoted to the method of preparation and the mode of testing of a platinized surface free from electrolytic polarization and, as far as possible, electrochemically reversible.

The dielectric properties of solutions of magnesium sulphate have been measured, at low frequency (1000 cyc./sec.), over a range of dielectric constant from 37 to 110. The solvents were mixtures of dioxan and water (from 0 to 50 % dioxan) and solutions of urea in water (up to 9·0 M urea).

The experimental data may be interpreted in terms of the Bjerrum theory of ion association, after due allowance has been made both for the Debye-Falkenhagen effect of relative displacement of ion and ion atmosphere, and also for the solvent dipole saturation effect postulated by Sack. Reasonably steady values for the thermodynamic association constants (K_α) are obtained in each solvent. The values of K_α indicate that the mean diameter of the ion pairs is not a constant, independent of the dielectric constant of the solvent; the diameter apparently falls from 5·8 A, in 50 % dioxan, to about 3·7 A in 9 M urea.

The degree of orientation of dipoles by the ionic charges (the Sack saturation effect) increases with falling dielectric constant of the medium. Possible reasons for these results are discussed.

This work supports the view that the behaviour of electrolytes is to be interpreted solely in terms of simple Coulomb forces; it is not necessary to invoke any specific effect due to the chemical nature of the solvent.

The soft X-ray spectroscopy of solids. By H. W. B. SKINNER. (*Communicated by A. M. Tyndall, F.R.S.—Received* 25 *January* 1940.)

The present paper is the first of a series which aims at the determination of the level systems of substances in the solid state, in a way as far as possible analogous to the determination of the level system of an atom by spectroscopic information on substances in the gaseous state. It is shown that, by the use of soft X-ray spectra, in the region 40–500 A, emitted from the anticathode in an X-ray tube, this object is, to a large extent, attained in the case of elements; I hope subsequently to deal with the case of chemical compounds. The general technique for obtaining such spectra, free from contamination, is described. The effect of varying the temperature of the substance has been investigated and, in the case of a metal, is shown to be directly related to the Maxwellian distribution of the conduction electrons. Thermal expansion of the substance can also be detected spectroscopically. Several general characteristics of the level system of a solid have been established directly; in particular, the fundamental difference between the systems, metals and insulators. A discussion is given of the validity of the detailed information supplied, and the conclusion is reached that it is almost entirely reliable. Thus the experimental work also provides data for a detailed analysis of the level system of a particular solid, containing only atoms of one type, in terms of the crystal structure and of any other factors which may prove to be important.

The microcoulomb experiment. By F. EHRENHAFT. (*Communicated by A. Einstein, For.Mem.R.S.—Received* 25 *January* 1940.)

The present paper deals with the determination of the size and charge of single submicroscopic particles, measured in a small horizontal condenser of a diameter of approximately 8 mm. using the author's method, first stated in April 1910. To obtain the highest sensitivity solid particles (spheres) were used of an order of magnitude of 3×10^{-5} cm. and found charges less than that of the electron. Various objections were raised against his findings; namely, that the particles investigated were not spheres, and their density was not that of the macroscopic substance, so that his findings of charges less than the electronic charge were merely imaginary. I have succeeded in precipitating and measuring a particle after having observed it in the condenser under normal, and also very high gas, pressures. The particles were shown to be perfect spheres. A new method is indicated whereby it is possible to determine the size of submicroscopic spheres by pushing together two equal-sized spheres with a micromanipulator. The two diffraction disks overlap and render it very easy to determine the actual size. It is also possible to show that the density of each individual sphere is that of the macroscopic substance, since it can be computed easily from the velocity of fall of high gas pressures (according to Stokes's law). These two results being accurately determined, the author still found charges considerably less than the electronic charge; further, the deviations cannot be accounted for by errors of observation since the deviations are too great. The reason for using a solid substance (red amorphous selenium) and not oil, a liquid, was in the first place that liquids evaporate and therefore do not yield correct results, and secondly, that liquid spheres are not certain to preserve their sphericity when precipitated.

Thus charges less than that of the electron are to be considered as actually existing.

It has been noted that the charge, less than the electronic as carried by a single particle, is obtained without any assumption about the atomistics (quantization) of electricity or matter. All the other methods of determining the elementary charges yield only mean values, and even those result merely from the combination with certain atomic constants, namely, N, the Avogadro number, Planck's constant h, and the electronic mass m.

The thermal decomposition of gaseous benzaldehyde.

By C. N. HINSHELWOOD, F.R.S. and R. E. SMITH. (*Received* 27 *January* 1940.)

The thermal decomposition of benzaldehyde, which takes place predominantly according to the equation $C_6H_5CHO \rightarrow C_6H_6 + CO$, is homogeneous, and differs from the corresponding reaction of acetaldehyde in that it is of the first order above 100 mm., and is subject to partial inhibition by nitric oxide. The residual inhibited reaction shows a pressure dependence very like that of the normal acetaldehyde reaction. The activation energy increases as the pressure decreases.

The results can be explained by assuming the superposition of a chain reaction and of a molecular reaction. The order of the uninhibited reaction and the influence of nitric oxide at different initial pressures of benzaldehyde indicate the probable mechanism of the chain part of the decomposition, which appears to involve the decomposition of the radical CHO as an essential step.

Dielectric loss in simple alkyl resins.

By D. R. PELMORE and E. L. SIMONS. (*Communicated by E. V. Appleton, F.R.S.—Received* 1 *February* 1940.)

The paper describes the results of measurements of dielectric losses and their variation with temperature for some simple alkyl resins prepared by the condensation of $\alpha\omega$-dibasic acids and $\alpha\omega$-glycols.

The polymers are known to consist of long chains of the form

$$-[-CO-(CH_2)_x-CO-O-(CH_2)_y-O-].$$

It is found that when x and y are both even, the maximum value of dielectric loss is much smaller than when one or both of them are odd. This is attributed to the fact that when x and y are both odd the components of the dipole groups transverse to the chain are all in the same direction; when one of them is odd and the other even they alternate in pairs, and when both are even the direction of the dipoles alternates so that any one dipole is situated between two others whose sense is in the opposite direction.

The work was suggested by the results of previous work using dibasic esters mixed with paraffin wax (*Proc. Roy. Soc.* A, 1939, **172**, 502), in which it was found that the loss was either negligible or positive according as the number of carbon atoms between the two ester groups was even or odd.

The behaviour of polar molecules in solid paraffin wax.

By D. R. PELMORE and E. L. SIMONS. (*Communicated by E. V. Appleton, F.R.S.—Received* 1 *February* 1940.)

The thermal explosion of diethyl peroxide.

By E. J. HARRIS. (*Communicated by A. C. G. Egerton, Sec.R.S.—Received* 12 *February* 1940.)

The critical pressure at which the slow decomposition of diethyl peroxide gives place to explosive reaction has been measured at different temperatures and in various vessels. On the basis of the theory of thermal explosions (Semenoff, Frank-Kamenetzky) a product $P=\frac{Qk}{X}\cdot\frac{E}{RT^3}\cdot\frac{p}{76}\cdot r^2\frac{273}{22{,}400}$ is found, which for a sphere should be 3·82, when the pressure is the limiting value at which explosion just takes place. The experimental values of the product was about 70 % of this value, and similarly the use of cylindrical vessels led to a result lower in the same ratio. The plot of $\log p/T^3$ against $1/T$ for a given vessel falls on a line of slope E, the E approximating to that found for the unimolecular slow reaction, which is in accord with the above as $k = ze^{-E/RT}$. The conductivity X, if increased by addition of hydrogen or helium, does not alter p (the critical pressure) in proportion, which may be due to inefficient energy transfer. The same considerations are applied to the data of Rice *et al.* concerning azomethane and ethyl azide.

The induction period of the explosion is shown to fit Todes's formula. The heat of decomposition was calculated from the heat of combustion.

Structural analysis of *Oenothera* complexes.

By D. G. CATCHESIDE. (*Communicated by F. T. Brooks, F.R.S.—Received* 14 *February* 1940.)

The fourteen end-pairing segments of *Oenothera* chromosomes have been defined in terms of their property of pairing at meiosis and the ways in which they are combined in the chromosomes of different gametic complexes. The comparison of a number of different complexes has led to the following conclusions concerning their structure in respect of the end segments of the chromosomes:

^{h}*Hookeri*	1·2	3·4	5·6	7·8	9·10	11·12	13·14
flavens	1·4	2·3	5·6	7·8	9·10	11·12	13·14
velans	1·2	3·4	5·8	6·7	9·10	11·12	13·14

excellens	1·2	3·4	5·6	7·10	8·9	11·12	13·14
[b]*franciscana*							
[b]*purpurata*							
[a]*blandina*	1·2	3·4	5·6	7·10	8·13	9·14	11·12
blandina-A	1·2	3·11	4·12	5·6	7·10	8·13	9·14
blandina-B	1·4	2·3	5·6	7·10	8·13	9·14	11·12
latifrons	1·2	3·4	5·6	7·10	11·12	8·14	9·13
rubricalyx-α	1·2	3·4	5·6	7·14	8·13	9·10	11·12
[b]*decipiens*							
rigens	1·2	3·4	5·6	7·12	8·14	9·10	11·13
gaudens rubens	1·2	3·11	4·9	5·6	7·12	8·14	10·13
flectens	1·4	2·3	5·7	6·10	8·9	11·13	12·14
albicans	1·4	2·14	3·6	5·7	8·9	10·12	11·13
curvans	1·14	2·3	4·6	5·13	7·11	8·9	10·12

The value of the analysis in relation to evolutionary and genetic studies of these plants is pointed out. The three European species *Lamarckiana*, *suaveolens* and *biennis* are related in origin; each of the first two of these has a complex in common with *biennis*, but a different one in each case. The common complexes are identical in gross structure and very similar in gene content. A preliminary genetic map of *Lamarckiana* has been drawn and a complication in the origin of new alethal complexes from *Lamarckiana* laid bare.

Nerves controlling the tone of the alimentary canal in the earthworm.

By N. Millott. (*Communicated by H. Graham Cannon, F.R.S.—Received 15 February* 1940.)

The alimentary canal of *Lumbricus* exhibits autonomous rhythmic movements co-ordinated by nerve elements situated in its walls.

The gut is subject to indirect control by extrinsic nerves which fall into two groups, mutually antagonistic in their effects. Nerves which augment the tone of the gut muscles leave the central nervous system by the middle and posterior nerves of each segment and join a plexus of nerve elements situated in the peritoneum of the body wall from which nerves pass to enter the gut by the ventro-lateral regions of each septum. Nerves which diminish the tone of the gut leave the central nervous system by the anterior, middle, and posterior nerves of each segment and join a plexus of nerve elements situated in the muscular layer of the body wall, from which nerves arise which enter the gut via the dorso-lateral regions of each septum.

By the uses of nicotine both augmentor and inhibitor nerve pathways are shown to include nerve cells. The action of sympatheticomimetic and parasympatheticomimetic drugs on the gut of *Lumbricus* is discussed, and evidence is presented showing that the augmentor nerves are cholinergic, and the inhibitors possibly adrenergic. A substance with the pharmacological properties of acetyl-choline can be extracted from the tissues of the earthworm. Another substance capable of destroying acetylcholine (choline esterase) is shown to exist in the earthworm.

The alimentary canal receives another nerve supply from the peripharyngeal commissures of the central nervous system by means of a number of fine plexiform nerve elements partially embedded in the pharyngeal musculature of either side ("pharyngeal plexus"). The anatomical relationships of these nerves are described.

The pharyngeal plexuses are shown to embody nerve elements which also exert an indirect control over the gut. Stimulation of these nerves results in diminution of the tone of all regions of the gut behind the pharynx.

The nerve supply of the gut is compared with that which is known in other annelids, and the mechanism of indirect control is compared with that existing in vertebrates.

The significance of the results in relation to recent work on chemical transmitters in invertebrates, and to the problem of the evolution of the autonomic nervous system, is discussed.

Studies on the transmission of sugar-beet yellows virus by the aphis *Myzus persicae*.

By M. A. WATSON. (*Communicated by Sir John Russell F.R.S.—Received* 15 *February* 1940.)

The efficiency of the vector *Myzus persicae* in transmitting sugar-beet yellows virus increased greatly with increasing feeding time on the infected plants. Infection was produced on a succession of healthy plants for 1, 2, or 3 days depending on the length of the infection feeding time. The infectivity of the vectors increased with increasing feeding time on the healthy plants undergoing infection, and decreased with increasing feeding time on healthy plants prior to those on which the infection trial was made. There was no clearly defined "incubation period" of the virus in the vector, below which no insect could cause infection, but there was variation in the time between cessation of infection feeding of the aphis and the initiation of infection in the healthy plants.

The relations of this virus with its vector differ from those of the viruses already described as *non-persistent*. For the latter viruses infectivity is lost by *M. persicae* soon after cessation of infection feeding. After fasting the vectors become optimally infective almost immediately on penetrating infected tissues of the leaf. Their infectivity decreases with increasing feeding time on the infected plants, and increases only slightly with increasing feeding time on the healthy plants.

The behaviour of sugar-beet yellows virus is compared with that of curly-top virus of sugar beet, in which infectivity also persists for an indefinite period in the vector and increases with increasing feeding time on infected and healthy plants.

ABSTRACTS

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10 April 1940

The electric strength of solid dielectrics in relation to the theory of electronic breakdown.

By A. E. W. Austen and S. Whitehead. (*Communicated by N. F. Mott, F.R.S.—Received* 19 *December* 1939.)

Methods are described by which the "intrinsic" electric strength of solid dielectrics may be defined and evaluated. It is shown that the magnitudes, and the effect, temperature and thickness upon the electric strengths of certain crystals agree with Fröhlich's theory of electronic breakdown, as also does the effect of disordered structure and microstructure in similar instances. On the other hand, departures from theory occur with complex organic dielectrics and also with crystals when, with the latter, certain limits, e.g. of temperature, are exceeded. Some observations are made on the effect of temperature and electric stress upon the conductivity.

Observations on the structure, development and electrical reactions of the internal ear of the shaker-1 mouse (*Mus musculus*).

By H. Grüneberg, C. S. Hallpike and A. Ledoux. (*Communicated by E. D. Adrian, F.R.S.—Received* 2 *February* 1940.)

Histological examination of the post-foetal development of the internal ear in normal and shaker mice was carried out at periods between 9 hours and 12 days after birth.

The normal process of post-foetal development appeared to pursue an identical course in both varieties and to be completed in 12 days.

Tests of hearing, measurements of the electrical response of the cochlea and histological examination of the internal ear were carried out in normal and shaker mice at

ages between 38 and 329 days. In all the shaker material deafness was found to be correlated with pathological changes confined to the scala media complex and gross reductions of the electrical response. The changes first appeared in the stria vascularis and Corti's organ and were progressive with age. Those in the spiral ganglion appeared later.

The degenerative process was thus shown to be initiated after birth in a fully differentiated organ, and comparison was made with similar results obtained by van Lennep in the Japanese waltzing mouse.

The parallel courses of the loss of the electrical response and of the degenerative changes in both the stria vascularis and in Corti's organ rendered the results indecisive as regards the relative merits of the "membrane" and "hair cell" theories of origin of the response.

A selective reduction of the response which occurred at high frequencies was correlated with some accentuation of the degenerative changes in Corti's organ at the base of the cochlea, and the resemblance of this to similar findings by Stevens, Davis and Lurie in the guinea-pig under different experimental conditions was discussed. The results were not considered to constitute a decisive contradiction of the membrane hypothesis.

No morphological abnormalities were found in the vestibular apparatus of the internal ear. It must therefore be supposed that the abnormal head movements are of extra-labyrinthine origin.

On the function of chlorocruorin. By R. F. EWER and H. MUNRO FOX, F.R.S. (*Received* 17 *February* 1940.)

The oxygen consumption of *Sabella* decreases soon after the concentration of dissolved oxygen in sea water falls below the value corresponding to air saturation, both at 10 and 17° C; above air saturation at 17° C there is no significant rise in oxygen consumption. The oxygen consumption of *Sabella*, whose chlorocruorin has been converted to carboxychlorocruorin, is lower than that of normal worms; this is the case at air saturation of the water and at oxygen concentrations below air saturation both at 10 and 17° C. The fall in oxygen consumption of the animals after treatment with carbon monoxide is not due to an action of the latter on cell enzymes. It follows that chlorocruorin functions as an oxygen carrier in *Sabella* at all temperatures and oxygen pressures to which the animals are subjected in nature.

The structure of the cell wall in some species of the filamentous green alga *Cladophora*. By W. T. ASTBURY and R. D. PRESTON. (*Communicated by Sir William Bragg, P.R.S.—Received* 21 *February* 1940.)

With a view to extending the work on *Valonia*, the cell walls of several species of *Cladophora* have been examined in detail by means of X-ray diffraction photographs and the microscope. The walls are found to consist of layers in which the cellulose chains in any one layer are inclined to those in the preceding and subsequent layers at an angle whose average is less than 90°. The two sets of striations on the layers of the wall correspond closely to the directions of the cellulose chains.

Each set of chains forms a spiral round the cell, and the spirals are of opposite sign. One tends to be flat and the other steep. On the whole, the steep spiral tends to become steeper on passing from the base of the filament to the tip, and the flat spiral flatter. In any one cell of the filament, the steep spiral is steepest at the end nearer the filament tip and the flat spiral flattest. Wherever such changes in inclination occur, the angle between the chains tends to remain constant.

It is suggested that the cell elongation is the factor causing the inclination of the steeper spiral to vary, and that the behaviour of the flatter spiral is best explained by the assumption of a protoplasmic mechanism causing a periodic change in the direction of cellulose chains through a constant angle. The development of a branch cell is reviewed and is found to proceed as the above suggestions would indicate.

Orientation of nuclear spins in metals. By H. Fröhlich and F. R. N. Nabarro. (*Communicated by N. F. Mott, F.R.S.—Received 22 February* 1940.)

In connexion with the possible use of nuclear magnetism for the magnetic cooling method, the behaviour of the nuclear spins of monovalent metals at very low temperatures is investigated theoretically. It is shown that, owing to the interaction between the nuclear spins and the conduction electrons, the nuclei of most monovalent metals should become ferromagnetic at temperatures of the order of 10^{-6} degree. The Curie temperature θ for this nuclear ferromagnetism is approximately given by

$$k\theta \sim \tfrac{1}{8}\epsilon^2/\zeta,$$

where ϵ is the hyperfine structure splitting of the free atom, and ζ is the energy region occupied by the conduction electrons. Temperatures of this order of magnitude should be attainable by an application of the magnetic cooling method.

The determination of the lifetime of active polymeric molecules. By T. T. Jones and H. W. Melville. (*Communicated by E. K. Rideal, F.R.S.—Received 26 February* 1940.)

A method of determining the distance of diffusion in space of a polymer reaction is described. It depends upon the fact that in a reaction—the direct polymerization of methyl acrylate—in which active molecules mutually destroy each other, this mutual interference may be demonstrated by using two sources of photo excitation. The velocity of polymerization then depends on the distance between the two sources. The exact theory of the method has been worked out for hydrogen atoms. The results obtained from this investigation are applied to the methyl acrylate reaction. This result is then further employed, along with previous data, to compute the absolute value of the reaction velocity coefficient between active polymer molecules and monomer.

The glide elements of body-centred cubic crystals, with special reference to the effect of temperature. By E. N. da C. Andrade, F.R.S., and Y. S. Chow. (*Received* 9 *March* 1940.)

The glide elements of single crystals of the body-centred cubic metal, sodium, have been determined at various temperatures. The glide direction is in all cases [111], but the glide plane changes successively from (112) to (110) to (123) as the temperature goes from − 185 to 20° C. It is shown that this change of glide plane with temperature is a particular case of a general rule to which body-centred metals conform: all three planes are possible glide planes but which is operative at any particular temperature is determined by the temperature referred to the melting point of the particular metal as standard. The case of iron, for which all three planes seem to be operative at once, is considered separately.

The critical shear stress has been determined for sodium, and shows a small variation only over the temperature range − 185 to 20° C.

Experiments with sodium, iron and molybdenum go to show that the spacing of the glide planes increases markedly as the temperature is raised.

It has been found that, for equal strain, the crystallite rotations, as evidenced by the asterisms, are much greater at low temperatures, where the hardening is greater, which goes to support the view that hardening is intimately connected with the rotation of crystal fragments. The breaking up of the asterisms into discrete spots which has been found with sodium and potassium is shown to be due to recrystallization.

The general implications of the results are discussed.

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16 May 1940

Thermal ionization of strontium.

By B. N. Srivastava, D.Sc. (*Communicated by M. N. Saha, F.R.S.—Received* 4 *March* 1940.)

In this paper the thermal ionization of strontium vapour has been experimentally investigated by an apparatus already described by the author elsewhere in his work on barium. Experiments have been carried out at various temperatures and pressures of strontium vapour, and the equilibrium concentrations of Sr^+ and electrons inside the furnace have been obtained by allowing them to effuse out through a narrow opening. From these the equilibrium constant and the energy of ionization have been calculated. The results obtained agree, within the limits of experimental error, with the theory of thermal ionization and the known spectroscopic value of the ionization potential of strontium.

The growth, growth energy and ageing of the chicken's heart.

By P. B. Medawar. (*Communicated by E. S. Goodrich, F.R.S.—Received* 7 *March* 1940.)

A property referred to as the *growth energy* of the chicken's heart, and representing the resistance of freshly explanted tissues to inhibition of their growth, is described and measured. The growth energy was found to decline exponentially with time over the range 6–18 days. It is shown that the growth energy is proportional to, and therefore a measure of, the specific growth rate of the heart *in vivo*. The growth in mass of the chicken's heart can therefore be described by a mean curve, which is that of a Gompertz equation. The growth energy of tissues rises sharply during the course of cultivation, and initial embryonic age differences are smoothed out. The growth of mesenchyme tissues is shown to be dependent upon the movement of their constituent cells. The relation between growth, movement, metabolism, and differentiation is discussed, and a theory proposed which indicates the degree of their functional dependence upon one another. The relevance of the Gompertz equation to problems of relative growth and senescence is briefly considered.

Ocular interaction in its relation to measurement of brightness threshold.

By B. H. Crawford. (*Communicated by Sir John Parsons, F.R.S.—Received* 11 *March* 1940.)

It is found that in all cases binocular vision produces a lower threshold than monocular vision, but, under static conditions of foveal vision at all brightness levels and of parafoveal vision at very low levels, the difference is small and can be wholly explained by the fact that with two eyes there is a greater probability of the test field being seen than with one eye. For parafoveal vision under static conditions at medium and high brightnesses binocular vision gives a threshold about 30% lower than does monocular vision. When the state of adaptation is changing—after cutting off a conditioning field of high brightness—both foveal and parafoveal retinal areas show more rapid recovery for binocular than for monocular vision. In the fovea the difference is greater in the initial stages of recovery of dark adaptation, while in the parafovea the difference is approximately constant during the whole process.

It is also found that when one eye is used for making threshold measurements it is completely unaffected by exposure of the other eye to fields of brightness up to 130 candle/ft.^{2}.

Any subsidiary pupil diameter effects are avoided by the use of Maxwellian view of the visual field.

On a green helical organism and its motion.

By L. E. R. Picken. (*Communicated by J. Gray, F.R.S.—Received* 13 *March* 1940.)

A green helical organism is described which displays a new type of movement. The helix moves by growing at the anterior end and diminishing posteriorly; it does not rotate.

Observations on the change in shape produced by dehydration show that the structure of the organism is markedly anisotropic. By analogy with other fibrous structures, it may be concluded that anisodiametric micells or molecules are orientated parallel to the long axis.

It is pointed out how the growth and activity of the organism are related to this axis.

The systematic position of the organism is discussed and its possible affinity with the Spirochaetoidea (Dobell) and Cyanophyceae considered.

Experiments with a velocity spectrometer for slow neutrons.

By G. E. F. Fertel, D. F. Gibbs, P. B. Moon, G. P. Thomson, F.R.S. and C. E. Wynn-Williams. (*Received* 14 *March* 1940.)

A velocity spectroscope for slow neutrons has been constructed upon the principle of measuring the times of transit of individual neutrons from an intermittent source to a distant ionization chamber.

Measurements have been made of the velocity distribution of the slow neutrons from a paraffin-wax howitzer at room temperature.

By the interposition of absorbers, the adsorption coefficients of boron and cadmium have been measured for neutrons of different velocities. The results for boron are inconsistent with the law of inverse velocity.

Radiative processes involving fast mesons.

By F. Booth and A. H. Wilson. (*Communicated by R. H. Fowler, F.R.S.—Received* 19 *March* 1940.)

The general theory of the interaction of mesons with an electromagnetic field is developed, starting from Kemmer's matrix formulation of the meson equations. The general theory is applied to the scattering of light by mesons, the emission of radiation by mesons during collisions and the production of pairs of mesons. A brief discussion of the experimental results on the energy losses of "heavy electrons" in cosmic rays is given, and it is shown that the experiments are consistent with the view that the heavy electrons have spin 1 and that the theory of the interaction of mesons with an electromagnetic field is probably valid for energies considerably greater than the rest energy of a meson.

A magnetic study of the two phase iron nickel alloys.

By A. T. Pickles and W. Sucksmith, F.R.S. (*Received* 20 *March* 1940.)

Measurements of magnetic saturation intensity of annealed iron-nickel alloys have been used to demonstrate the existence of a two phase field in the iron-rich part of the system. The phase boundaries have been determined at temperatures above 450° C, and the relation of the equilibrium phase diagram to the thermal hysteresis observed under normal rates of temperature has been studied.

Explosion waves and shock waves. VI. The disturbance produced by bursting diaphragms with compressed air.

By W. Payman and W. C. F. Shepherd. (*Communicated by Sir Jocelyn Thorpe, F.R.S.—Received* 21 *March* 1940.)

Experimental verification of the theoretical relationships governing the motion of shock waves has been derived from an investigation into the development of the

disturbance set up in a uniform tube when a body of compressed gas, confined at one end by means of a copper diaphragm, is released by rupture of the diaphragm. The wave-speed camera has been used to obtain continuous Schlieren records of the passage of these effects along the tube and to analyse their properties under a variety of experimental conditions, the main variations being those caused by the use of different thicknesses of diaphragm, different lengths of compression and expansion chambers, and of different gases in these chambers.

The pressure effects released on the rupture of the diaphragm are the shock wave, a vortex formation and the expanding gases from behind the diaphragm. Schlieren snapshot (spark) photographs have been obtained showing the structure of these pressure effects when they are projected from the end of the tube into free air. The photographs show, in addition to these details, the establishment of a system of stationary sound waves.

Diagrams have been constructed based on the experimental data showing the apparent form of the wave during various phases of its progress.

A mathematical analysis of the distribution in maize of *Heliothis armigera* Hübner. By M. G. Walker. (*Communicated by W. R. Thompson, F.R.S.—Received* 28 *March* 1940.)

The distribution among maize plants of the eggs of the American boll-worm, *Heliothis armigera* Hübner, is discussed and analysed.

The problem is considered in relation to what is known of the connexion between the state of development of maize plants and their attractiveness to ovipositing boll-worm moths. The actual frequency distribution of the eggs suggests a random as opposed to a uniform distribution, but it is shown that the conditions required for a pure mathematical random distribution cannot be satisfied. Because the maize plants differ from one another in absolute degree of attractiveness at any one time, and in relative degree of attractiveness with the passing of time, it is not true that every plant has the same chance of receiving any given egg.

It is demonstrated that a mathematical theory, which is eventually one of random distribution, but which incorporates a modification to allow for the varying degrees of attractiveness of the plants, gives a fairly good representation of the egg distribution found in the field.

Theoretical distributions to fit the data are calculated by two methods. One is a discontinuous process which is presented as only a rough approximation of what it is intended to express. The other uses the compound Poisson series of Greenwood and Yule. The continuous variation in nature both in space and time, which is the essential difficulty of the problem, is discussed.

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